Analysis of the Epstein-Barr virus transcription factor, Zta.

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I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree.

Signature:……

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Summary

Epstein-Barr virus (EBV) is a ubiquitous pathogen that infects most of the adult population and persists for life after the initial contact. The extreme success of the virus is attributed to its bipartite life cycle, which consists of a dormant-like state of latency, with periodical reactivation to the virus producing, lytic phase. Zta (BZLF1, Z, Zebra or EB1) is a multifunctional viral protein that belongs to the bZIP family of transcription factors and is known as the master lytic regulator of EBV. Together with transcriptional activation, Zta has been shown to be involved in DNA binding-dependent transcriptional repression, particularly of the host class II major histocompatibility complex transactivator, CIITA. Distinct protein domains, as well as various post-translational modifications, like phosphorylation of Serine 209 by the viral protein kinase (VPK), have been linked to different functional roles of Zta.

In the present study, it was shown that VPK can partially inhibit SUMOylation of Zta on Lysine 12, in a manner which was not dependent on Serine 209 phosphorylation. However, no direct interaction of VPK and Zta could be observed and no significant effect of either proteins on histone H2AX phosphorylation was seen.

Interestingly, *in vitro* reporter assays revealed that fusion of a SUMO moiety to the amine-terminus of Zta inhibited repression of the *CIITA* promoter, but not the activation of the viral *BHLF1* promoter, pointing at divergent mechanisms of action of transcriptional repression and activation by Zta. Moreover, truncation of the carboxy-terminal dimerisation domain of Zta (crucial for protein-DNA interaction) abrogated *BHLF1* transactivation but not *CIITA* down-regulation, revealing underlying differences in DNA binding requirements for the two processes.

Further *in silico* sequence analysis, coupled with a mutation approach of the *CIITA* promoter, confirmed that an alternate route to the Zta DNA binding-dependent repression exists. Finally, no single promoter element could be linked to down-regulation of *CIITA*, suggesting sequestration of a possible, yet unknown cofactor, by Zta.

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53BP1	p53-binding protein 1
AA	Antibiotic and antimycotic
AIDS	Acquired immunodeficiency syndrome
ARE	Activation response element
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BART	BamHI rightward transcripts
BCL-2	B cell lymphoma 2 (protein)
BCR	B cell receptor
BL	Burkitt's lymphoma
BRA	Basic region A
BRB	Basic region B
bZIP	Basic leucine zipper
C/EBPα	CCAAT/enhancer binding protein α
cAMP	cyclic adenosine monophosphate
CAT	Chloramphenicol acetyltransferase
CBP	CREB-binding protein
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation coupled with sequencing
CIITA	Class II major histocompatibility complex transactivator
CMV	Cytomegalovirus
Cp	Latency promoter within BamHI C digestion fragment

CpG	Cytosine-phosphate-Guanine
CR2	Complement receptor type 2
CRE	cAMP responsive element
CREB	cAMP response element-binding protein
CT	Carboxy terminus
CTL	Cytotoxic T lymphocyte
DDR	DNA damage response
DMEM	Dulbecco's modified Eagle's minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DPBS	Dulbecco's phosphate buffer saline
dsDNA	Double-stranded deoxyribonucleic acid
DTT	Dithiothreitol
EBER	Epstein-Barr virus-encoded ribonucleic acid
EBNA	Epstein-Barr viral nuclear antigen
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EICE	Ets/ISRE-consensus element
EMSA	Electrophoretic mobility shift assays
FCS	Foetal calf serum
Fp	Lytic promoter for *EBNA1*
GFP	Green fluorescent protein

GST	Glutathione S-transferases
GTP	Guanosine triphosphate
H3K27ac	Histone H3 acetylated at Lysine 27
H3K27me3	Histone H3 trimethylated at Lysine 27
H3K9ac	Histone H3 acetylated at Lysine 9
H3K9me3	Histone H3 trimethylated at Lysine 9
HDAC3	Histone deacetylase 3
HEK	Human embrionic kidney (cells)
HeLa	Henrietta Lacks (cells)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHS-6	Human herpesvirus 6
HHS-7	Human herpesvirus 7
HL	Hodgkin’s lymphoma
HRP	Horseradish peroxidase
HSS	Hypersensitive site
HSV-1	Herpes simplex virus-1
HSV-2	Herpes simplex virus-2
ICTV	International Committee on Taxonomy of Viruses
IL-10	Interleukin-10
IM	Infectious mononucleosis
INF-γ	Interferon gamma
IR	Internal repeats
IRF3	Interferon regulatory factor 3
ISRE	Interferon stimulated response element

KSHV	Kaposi's sarcoma-associated herpesvirus
LB	Luria-Bertani
LCL	Lymphoblastoid cell lines
LMP	Latent membrane protein
MEF-2	Myocyte enhancer factor-2
MHC	Major histocompatibility complex
miRNA	Micro ribonucleic acid
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
NLS	Nuclear localisation signal
NPC	Nasopharyngeal carcinoma
NT	Amine terminus
OHL	Oral hairy leukoplakia
ORC	Origin recognition complex
ORF	Open reading frame
oriLyt	Origin of lytic replication
oriP	Origin of plasmid replication
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PIPES	2-ethanesulfonic acid
PKC	Protein kinase C
PLB	Passive lysis buffer
PML	Promyelocytic leukemia

PMSF	Phenylmethylsulfonyl fluoride
PPI	Phosphatase inhibitor
PSB	Protein sample buffer
PSG	Penicillin, Streptomycin, L-Glutamine
PTLD	Post-transplant lymphoproliferative disorder
PTM	Post-translational modification
Qp	Latency promoter within BamHI Q digestion fragment
qPCR	Quantitative polymerase chain reaction
RE	Restriction enzyme
RNA	Ribonucleic acid
Rp	Promoter of *BRLF1*
RPMI	Roswell park memorial institute medium
RT	Room temperature
SDS	Sodium dodecyl sulfate
SNP	Small nucleotide polymorphisms
ssDNA	Single-stranded deoxyribonucleic acid
SUMO	Small Ubiquitin-like modifier
SV40	Simian virus 40
TA	Transactivation domain
TR	Terminal repeats
TSS	Transcription start site
UR	Unique region
VPK	Viral protein kinase (EBV)
VZV	Varicella-zoster virus

WB	Western blot
Wp	Latency promoter within BamHI W digestion fragment
XBP-1	X-box binding protein 1
XLP	X-linked lymphoproliferative syndrome
ZIP	Leucine zipper
ZKO	Zta knockout
Zp	Promoter of *BZLF1*
ZRE	Zta response element
γHV68	Murine γ-herpesvirus 68

1. Introduction.

1.1 Herpesviruses.

Herpesviruses are ubiquitous infectious agents that are found across a wide range of various species. According to ICTV (International Committee on Taxonomy of Viruses), until recently, all herpesviruses were classified into the family of *Herpesviridae* with 3 subfamilies of *α*-, *β*- and *γ-herpesvirinae*, that included viruses occurring in mammals, birds and reptiles. These subfamilies differ from each other by their ability to establish latency in different tissues: *α-herpesvirinae* in neurons, *β-herpesvirinae* in monocytes and *γ-herpesvirinae* in lymphocytes (Davison, 2007). However, because of their omni-presence and apparent similarities with other species of viruses, recently an entire new Order (the highest taxonomic rank for viruses) called *Herpesvirales* was created; and to the existing family of *Herpesviridae*, two more were added, *Alloherpesviridae* and *Malacoherpesviridae*, which now encompass viruses that also infect amphibians, fish and the invertebrate bivalves (Davison, 2010; Davison et al., 2009). Nevertheless, the list of known herpesviruses is still considered far from complete. In addition to that, it is widely believed that the number of current species represents just a small fraction of the total that ever existed. At least in part, this is attributed to the fact that each herpesvirus is specific to one type of host, with which along the course of history they change and coevolve. On one hand this brings the risk of coextinction with the host species, but on the other, the benefit of extremely successful adaptability. Therefore, present-day her-pesviruses are among the most complex, successful and persistent viruses, with many species capable of establishing infection throughout the lifetime of the host (Arvin, 2007; Davison, 2002).

Herpesviruses complexity is reflected in their morphology. The virion particles are relatively large, averaging at about 200nm in diameter and are composed of at least four distinct parts [Figure 1.1] (McGeoch et al., 2006). The outer part is an envelope consisting of a lipid bilayer with associated viral glycoproteins [Figure 1.1 (1) and (2)]. Tethering of the virus to the cell surface is achieved through attachment glycoproteins, that in most cases bind to widespread cell membrane glycosaminoglycans (like heparan sulphate), receptors and integrins. Attachment alone is not sufficient for viral entry and is mediated through distinct tissue receptors, providing various herpesviruses with cell type specificity. Fusion glycoproteins, gB and gH/gL, that are responsible for the final stages of viral entry, on the other hand, are not species specific and are conserved across herpesviruses (Krummenacher et al., 2013). Immediately inside the envelope, is a matrix of diverse herpesviral encoded proteins, named the tegument, that have a wide range of functions [Figure 1.1 (3)]. Some of the tegument's important roles include establishment of initial viral infection by aiding transport to the nucleus, viral gene expression and immune evasion, as well as promoting latter stages of viral proliferation by assisting virion assembly and nuclear and cellular egress. In addition, the tegument proteins are an integral part of the virion architecture, binding both the viral envelope and the viral capsid (Guo et al., 2010a; Kelly et al., 2009). The herpesviruses capsid [Figure 1.1 (4)] takes the shape of a regular icosahedron (three-dimensional structure composed of 20 equilateral triangles) with the triangulation number (T-number) of 16, implying the total makeup of 162 multimeric structural proteins termed capsomers. One of the vertices of the icosahedron constitutes a dodecameric portal complex needed for viral genome ejection upon infection and encapsidation during virion production. The other 11 vertices are occupied by pentameric proteins and the remaining 150 capsomers

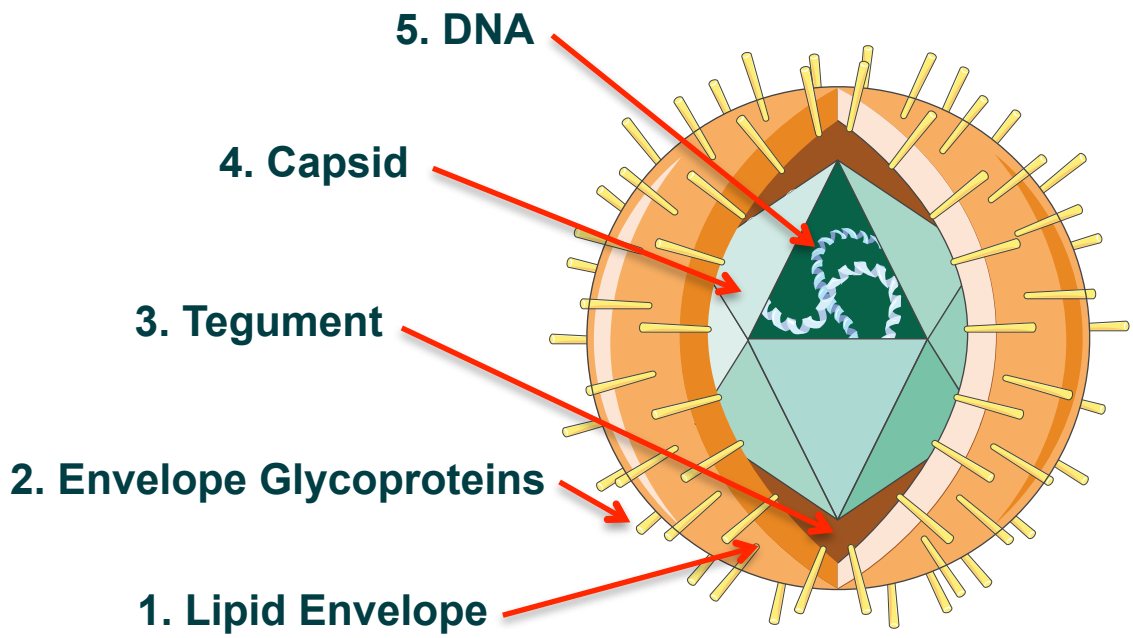


Figure 1.1 Graphical representation of a Herpesvirus virion particle.
Herpesvirus virion particles consist of: 1. Phospholipid membrane; with embedded 2. Envelope proteins; 3. The tegument; 4. Viral capsid; and the 5. DNA.

are hexamers of the same protein species (Brown and Newcomb, 2011; Mettenleiter et al., 2009). The main function of the capsid is to protect the viral genetic code until its release in the nucleus. In herpesviruses, the genome [Figure 1.1 (5)] is encoded by a linear double stranded DNA (dsDNA), up to 240kbp in length, made up of unique coding regions followed by repeats, which can be either direct, inverted, terminal or a combination of these. Moreover, the ends of the herpesviral DNA are marked by complementary overhangs, that aid circularisation upon reaching the nucleus (Arvin, 2007; McGeoch et al., 2006).

All herpesviruses have a distinct biphasic life cycle, which consists of lytic and latent stages. Initial contact with the host, usually causes a (primary) lytic infection. However, the resulting high load of viral replication is easily detected and eliminated by a healthy immune system. As viral proteins evoke an immune response, the host immune system causes a selection pressure for a small number of cells in which the virus has established a successful latent infection, enabling the infected cells to remain virtually undetectable. This is achieved through the expression of a minimal number of viral genes, immune evasion strategies, like interference with MHC class I and MHC class II antigen presentation, and genome replication only once during cell division, from a circular episome, during the latent phase. Thus, establishing latency, while intermittently reactivating to lytic cycle from a small number of infected cells, makes the host a reservoir of infection and contributes to the success and persistence of herpesviruses (Griffin et al., 2010; Wagner, 2008; Zuo and Rowe, 2012).

1.2 Epstein-Barr virus.

1.2.1 History of EBV discovery.

Epstein-Barr virus (EBV) belongs to the γ-herpesvirinae subfamily and is one of the eight known herpesviruses that infect humans: herpes simplex virus-1 (HSV-1), herpes simplex virus-2 (HSV-2), varicella-zoster virus (VZV), EBV, cytomegalovirus (CMV), human herpesvirus 6 (HHS-6), human herpesvirus 7 (HHS-7) and Kaposi's sarcoma-associated herpesvirus (KSHV). EBV is extremely widespread, with an estimated 90-95% of the total adult population infected, as the virus persists for life after the initial contact (Arvin, 2007). Despite this ubiquity, diseases related to EBV were not documented by medicine until the 1800s. The formal description of the virus was summarised only by the end of the century, first by a Russian physician, Filatov, in 1885 and then (independently) by a German physician, Pfeiffer, in 1889. However it was only in 1920 when the actual name for Infectious Mononucleosis (IM) (discussed in chapter 1.2.3) was given by Evans and Sprunt who identified it as a separate disease from acute leukaemia, though unable to link it to EBV or any other infectious agent. In 1932 another milestone was overcome with the discovery by Paul and Bunnell of an immunological test to check for IM. Nevertheless, until the 1960s attempts to find the cause of IM resulted in failure and the discovery of a new disease proved crucial for the breakthrough (Tselis and Jenson, 2006). Through the late 1950s and early 1960s, surgeon Denis P. Burkitt, who worked with patients in East Africa, published various articles on tumours occurring in African children, subsequently called Burkitt’s lymphoma (BL). The discovery of the new disease drew the attention of M. Anthony Epstein who requested some samples from Burkitt and by 1964, with the help of Achong and Barr, identified a new herpesvirus in BL tissue using electron microscopy (Epstein et al.,

1964). Isolation of the virus proved to be difficult, and Epstein turned to spouses Gertrude and Werner Henle who almost by chance found a link between EBV and IM. The serum of a technician (Elaine Hutkin) in their laboratory was used as negative control for EBV antibodies. However, once she developed IM, the serum started to react to the antibodies. After further investigation, in 1968 they showed that EBV indeed caused IM. Since then a vast array of different diseases have been linked to EBV (discussed in more detail in chapter 1.3) (Robertson, 2005; Tselis and Jenson, 2006).

1.2.2 EBV genome.

Like other herpesviruses, EBV has a linear dsDNA encoded genome, that circularises upon infection into a structure called the episome (Odumade et al., 2011). EBV has a relatively high (~60%) content of guanine and cytosine base pairings (compared to ~46% in humans (Romiguier et al., 2010)) and the length of the viral DNA can vary between different strains and types of EBV but is around 172kbp (Farrell, 2005). The genome is composed of 5 unique regions (UR 1-5), separated by four internal repeats (IR 1-4) and flanked by terminal repeats (TR) [Figure 1.2]. Different strains of EBV vary in the number of repeats and therefore the origin of an EBV infection can be traced using these DNA markers (Rickinson and Kieff, 2001).

In addition to strain variation, 2 different types of EBV have been identified: type 1 and type 2 (also known as type A and type B) (Rickinson et al., 1987). Individuals can be infected with either type of EBV, however, type 1 is more widespread, although type 2 has been shown to have a relatively high incidence in New Guinea and some equatorial African countries (Abdel-Hamid et al., 1992). The main difference between the two

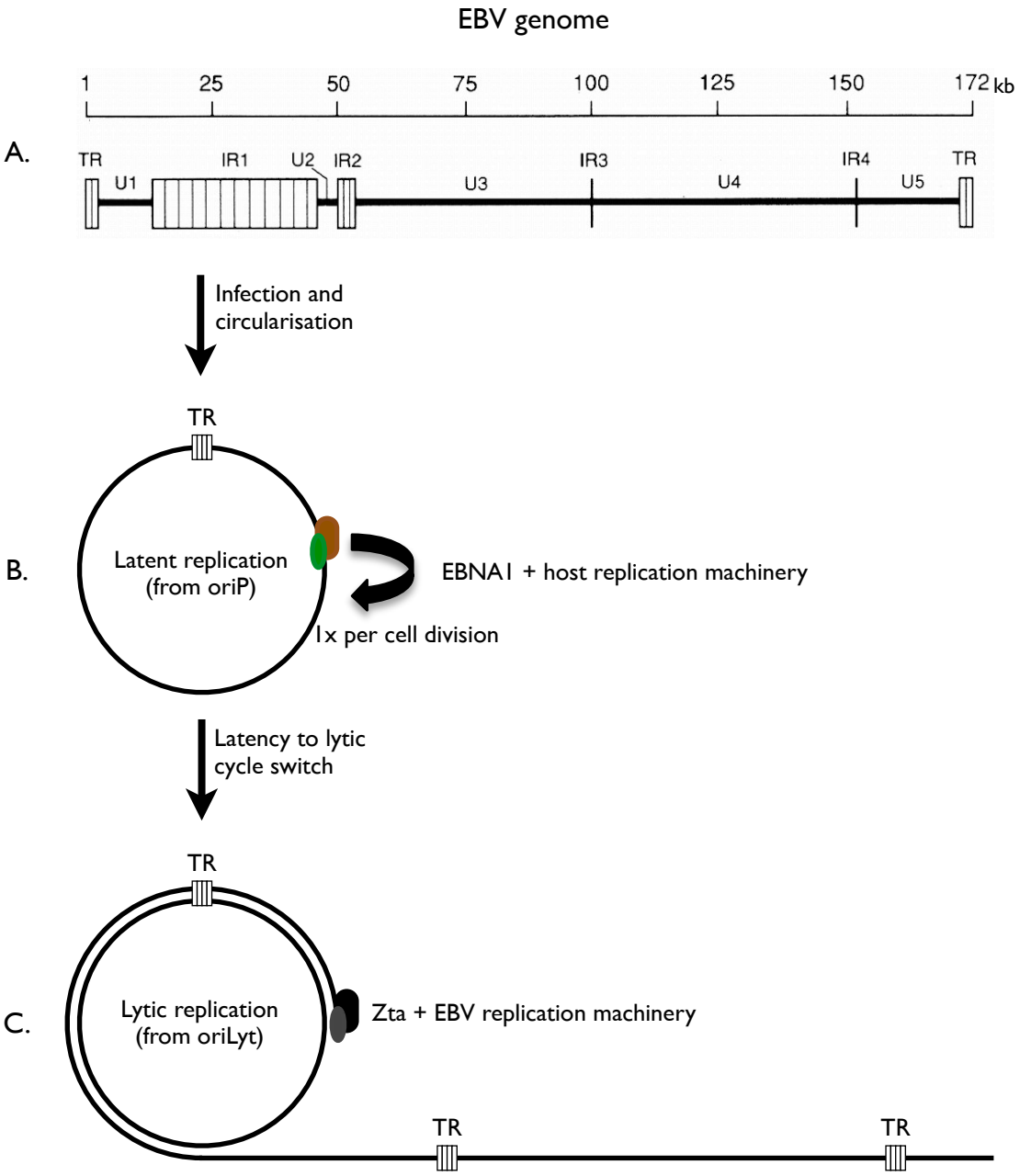


Figure 1.2 Schematic representation of the EBV genome.
A. Linear viral genome found in virions, with the relative position of terminal repeats (TR), internal repeats (IR 1-4) and uniques regions (U 1-5) is shown. Adapted from Straus et al. (1993) **B.** Latent viral genome replication depends on EBNA 1 protein and host replication machinery and happens only once per cell division with creation of two daughter episomes. **C.** Lytic EBV genome replication is dependent on Zta and viral DNA replication machinery, with creation of long concatameric DNA, that are cleaved and packaged into virions. Can be inhibited by acyclovir.

types of EBV is found in the *EBNA 2, -3A, -3B* and- *3C* (Epstein-Barr viral nuclear antigen 2, 3A, 3B and 3C) genes (Arrand et al., 1989; Sample et al., 1990). EBNA proteins are known to be important in EBV's ability to establish latent infection in B cells (Cohen et al., 1989; Tomkinson et al., 1993). Indeed, experiments in which B lymphocytes were transformed with EBV to generate *in vitro* lymphoblastoid cell lines (LCLs), showed that type 1 EBV is more competent at transforming and persisting than type 2 (Rickinson et al., 1987). Because of the immortalisation experiments, over the years, type 1 strain has been studied more, although type 2 is also found in cancers, indicating that *in vitro* immortalisation assays do not fully reflect the process of carcinogenesis in man. Type 1 was the first to be sequenced in 1984 by Baer et al. The nomenclature of EBV genes was based on the direction (rightward or leftward), number and location of each identified open reading frame (ORF) relative to BamHI DNA fragments, that were labelled alphabetically in a descending size order (Baer et al., 1984; Skare and Stominger, 1980). Thus a gene name like *BGLF4* can be deciphered as: BamHI Fragment G Leftward (transcript) 4; indicating that BamHI fragment G contains at least three more leftward transcripts. Some gene and protein names have been replaced by more meaningful ones over time, however the original nomenclature is still widely used, particularly for gene annotations. It is now known that the EBV genome expresses about a hundred mRNA transcripts that also include non-coding EBERs (Epstein-Barr virus-Encoded Ribonucleic acids) and the still poorly understood BARTs (BamHI rightward transcripts), the protein products of which remains to be found (Farrell, 2005). Some BARTs have been shown to code for micro RNAs (miRNAs) that can interfere with DNA replication (Pfeffer et al., 2004). EBERs on the other hand, were found to possess an anti apoptotic function, as well as the ability to interfere with several host enzymes,

however, much remains unknown about these transcripts (Clemens et al., 1994; Nanbo et al., 2002).

Upon entry and circularisation, the unmodified EBV genome undergoes extensive epigenetic alterations. One such modification is the Cytosine methylation of the CpG (Cytosine-phosphate-Guanine) motifs (Kintner and Sugden, 1981). In humans, CpG methylation has been linked to decreased promoter activity and gene silencing, usually through the recruitment of proteins that bind methyl-DNA and repress gene expression (Attwood et al., 2002; Di Croce et al., 2002). It is thought that DNA methylation of various viral genomes is an evolutionary adaptation aimed at decreasing viral gene expression and ultimately viral replication (Ernberg et al., 2011). Interestingly, EBV's co-evolution with the host has enabled the virus not only to overcome, but to use the DNA methylation to its advantage (Sinclair, 2013; Woellmer and Hammerschmidt, 2013). The viral transcription factor Zta, encoded by the *BZLF1* gene (discussed in more detail in chapter 1.5) has a unique property of being able to activate methylated promoters. In addition to that, a group of essential lytic genes are directly dependent on CpG methylation for efficient transcription. Therefore, the lack of EBV DNA methylation during initial infection prevents full lytic cycle activation and virion production (Bergbauer et al., 2010; Kalla et al., 2012; Kalla et al., 2010; Ramasubramanyan et al., 2012a; Woellmer et al., 2012). Latent promoters of *EBNA* genes (Cp, Wp, Qp), on the other hand, utilise the host's transcription machinery and are sensitive to methylation. This allows EBV not only to establish latency in an unmethylated genome environment, but also to gradually progress to more silent forms of latency as the Cp and Wp promoters become methylated (Paulson and Speck, 1999; Tierney et al., 2000). Since methylation is a pre-

requisite for successful lytic promoter activity, in its heavily methylated form, the viral genome is poised for re-activation and escape from latency (Sinclair, 2013).

Another form of epigenetic modification that EBV encounters upon infection is the genome packaging into chromatin, which consists of repeating units known as nucleosomes. The structure of a single nucleosome consists of protein octamers called histones, wrapped with approximately 147bp of DNA (Lodish et al., 2008). There are two distinct forms of chromatin: tightly packaged heterochromatin, and DNA accessible euchromatin. Different post-translational modifications (PTMs) of histones are associated with different chromatin states. Among the many, acetylation at Lysine 9 or Lysine 27 of histone H3 (H3K9ac and H3K27ac) is linked to euchromatin. Whereas, heterochromatin is known to have such modifications as Lysine 9 trimethylation of histone H3 (H3K9me3). Furthermore, polycomb group of proteins can be recruited by Lysine 27 trimethylation of histone H3 (H3K27me3), which is also associated with chromatin silencing (Lanzuolo and Orlando, 2012; Richards and Elgin, 2002). Investigation of various lytic promoter regions has shown that the heterochromatic H3K9me3 mark is present in the viral genes during latency. However, Zta can still bind to promoters containing H3K9me3 (Ramasubramanyan et al., 2012b). In addition to that, H3K27me3 polycoomb proteins associated mark was observed at various EBV genes during latency and did not decrease substantially during early lytic cycle. At the same time, viral lytic transcription from the studied promoters exhibited normal kinetics (Woellmer et al., 2012). Together these studies suggest that Zta can overcome various repressive marks and activate lytic promoters regardless of the chromatin state, although the mechanism by which Zta achieves this, remains unknown.

1.2.3 EBV primary infection.

The main route of EBV transmission is through the saliva, which is reflected in the common name for IM: kissing disease. It is thought that most EBV infections happen in infancy, since young children share various objects like cutlery and toys that might be contaminated with salivary secretions. At a young age the primary viral infection does not manifest itself symptomatically, however, as the individual matures to adolescence and then adulthood, there is a progressively higher chance of developing IM upon the first contact with EBV. The incubation period usually lasts around six weeks before IM starts to manifest. Among the most common symptoms of IM are fever, headaches, inflated lymph nodes and pharyngitis (throat inflammation), giving another common name for IM: glandular fever. IM is linked to the overreaction of the immune system to EBV infection in B cells, where the virus establishes latency III program with frequent reactivation to lytic replication [Figure 1.3]. This causes the production of abnormally high numbers of peripheral blood lymphocytes, particularly T cells, which in turn can lead to leukocytosis and anaemia (Kutok and Wang, 2006; Odumade et al., 2011).

EBV can infect both B cells and epithelial cells. The virus can be found in B lymphocytes of patients with such diseases as BL and Hodgkin's lymphoma (HL) and the mentioned above IM (Epstein et al., 1964; Niedobitek et al., 2000; Weiss et al., 1991). Epithelial cells are infected in such malignancies as nasopharyngeal carcinoma (NPC) or oral hairy leukoplakia (OHL) (Brooks et al., 1992; Greenspan et al., 1985). However, it is still a question of debate whether EBV infects epithelial cells in healthy individuals as well, and not just in the abnormal disease environment. The oro- and nasopharynx have a number of lymphoid tissues collectively called the Waldeyer's ring. The tonsils

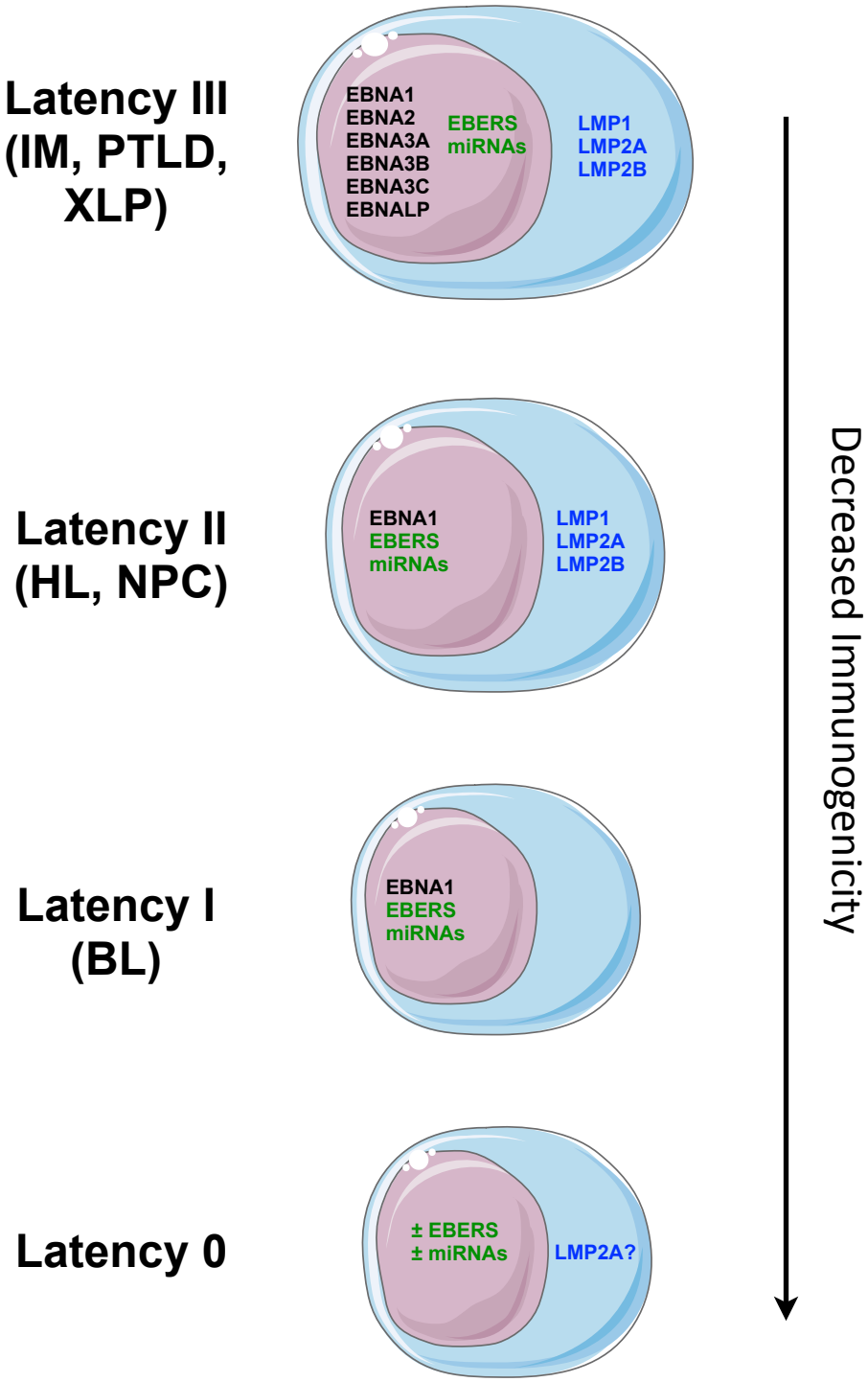


Figure 1.3 Differential expression of EBV latent genes in various latent programs.
As the EBV latency programs progress, less viral genes are expressed and the immunogenicity of infected cells decrease. Gene products expressed during different latency programs are shown. Some of the diseases associated with each latency program are indicated as well. IM: infectious mononucleosis; XLP: X-linked lymphoproliferative syndrome; PTLD: post-transplant lymphoproliferative disorder; HL: Hodgkin's lymphoma; NPC: nasopharyngeal carcinoma; BL: Burkitt's lymphoma.

that constitute the ring are composed of lymphoepithelial tissues, usually with invaginations called crypts. These protrusions create a large surface area for the tonsils, as they are the first line of immunological defence against exogenous pathogens (Hellings et al., 2000). Since the epithelium in the crypts can be as thin as a cellular monolayer, it is possible that EBV directly migrates to B cells between the cellular gaps or alternatively passes through epithelial cells without establishing infection, by a process called transcytosis. Nevertheless, the predominant model of primary EBV infection at the moment is that the epithelial cells become infected transiently and undergo rounds of lytic replication. This results in the production of a high number of viral particles, which can then infect naïve B cells in the oropharynx (Hutt-Fletcher, 2005; Tugizov et al., 2013).

Infection of B cells requires the binding of a number of cellular and viral receptors and subsequent endocytosis. The B cell specificity is mediated through the EBV glycoproteins gp350/220 that bind the cell surface receptor CD21 also known as complement receptor type 2 or CR2 (Tanner et al., 1988). Gp350 and gp220 are splice variants of the same viral gene *BBLF1* and are 350 and 220 kDa in size respectively. The smaller gp220 protein variant is 197 amino acids shorter than the 907 residues long gp350 but retains the CR2 binding domain (Beisel et al., 1985). To complete the fusion with the endocytic vesicle membrane, EBV utilises glycoprotein gp42 (*BZLF2* gene) that binds to major histocompatibility complex (MHC) class II and recruits other fusion glycoproteins gB, gL and gH (Kirschner et al., 2006; McShane et al., 2003). Epithelial cells, on the other hand, do not endocytose EBV and usually do not express a CR2 receptor, with exception of some tumour cell lines that were shown to have low levels of CR2. The biological significance of this observation *in vivo* still remains to be established. It is

now known however, that the BMRF2 protein can bind to β1 family of integrins and facilitate entry into epithelial cells (Fingeroth et al., 1999; Hutt-Fletcher, 2005; Xiao et al., 2008).

1.2.4 EBV latent phase.

Unlike their lytic counterparts, latent EBV genes do not have homologues in other herpesviruses and are unique to the virus. Latent cycle transcripts include six EBNA proteins, EBNA1, -2, -3A, -3B, -3C and -LP; three LMP proteins (latent membrane proteins), LMP1, -2A and -2B; as well as the non-coding BARTs, EBER1 and EBER2 RNAs. As EBV infects undifferentiated naïve B cells in the tonsillar crypts, the virus establishes latency III or 'growth program' during which the entire array of the aforementioned latent genes are expressed (Joseph et al., 2000). These viral products push the B cells to differentiate into active B cell blasts. Since the latency III is highly immunogenic due to the number of genes expressed, the immune response creates a selection for B cells in which EBV has established latency II or 'default program'. During latency II, the promoter for EBNA genes switches from Wp/Cp to Qp, causing the shut off of most EBNA proteins except EBNA1. B cell blasts are then further pushed into forming germinal centres in B cell follicles where they differentiate into long living memory B cells that circulate around the body, creating a reservoir for viral infection (Babcock et al., 2000; Casola et al., 2004; Yoshioka et al., 2003). In these cells the successful EBV infection is in a true latency form (latency 0 or just 'latency program'), during which almost all viral transcription switches off, with probable exception of LMP2A, BARTs and EBERs genes (Miyashita et al., 1997). When the resting memory B cells occasionally divide, latency I transcription is initiated, and EBNA1 is upregulated once again, to

allow the replication of the viral episome. Minimal protein expression during these later forms of latency allows the virus to avoid immune surveillance and persist for life [Figures 1.3 and 1.4] (Amon and Farrell, 2005; Hochberg et al., 2004; Thorley-Lawson, 2005).

Differential expression of EBV genes in various forms of latency is related to the function and immunogenicity of each protein, as the cytotoxic T lymphocyte (CTL) response creates a constant selective pressure on the infected cells. EBNA1 is present during most forms of latency as it is responsible for viral episome replication. It does this by binding to the origin of plasmid (i.e. latent) replication OriP and recruiting host origin recognition complex (ORC) that initiates DNA replication (Dhar et al., 2001). EBNA1 can also activate gene expression, including transcription from the Cp promoter (Kang et al., 2001). In addition to that, EBNA1 can inhibit the Qp promoter, autoregulating its own production. Unlike most latent proteins, EBNA1 possesses low immunogenicity due to Glycine-Alanine repeats in the protein sequence, that interfere with MHC class I presentation (Frappier, 2012).

The other EBNA proteins do not posses the origin binding ability, but can also modulate cellular and viral gene expression. Rather than directly binding to the DNA, EBNA 2, -3A, -3B, -3C and -LP interact with cellular factors to regulate protein synthesis (McClellan et al., 2012; Robertson et al., 1996). Amongst others, this includes EBNA2 upregulation of LMP proteins, which major function is to facilitate B cell differentiation in the absence of normal B cell maturation signals (Johannsen et al., 1995).

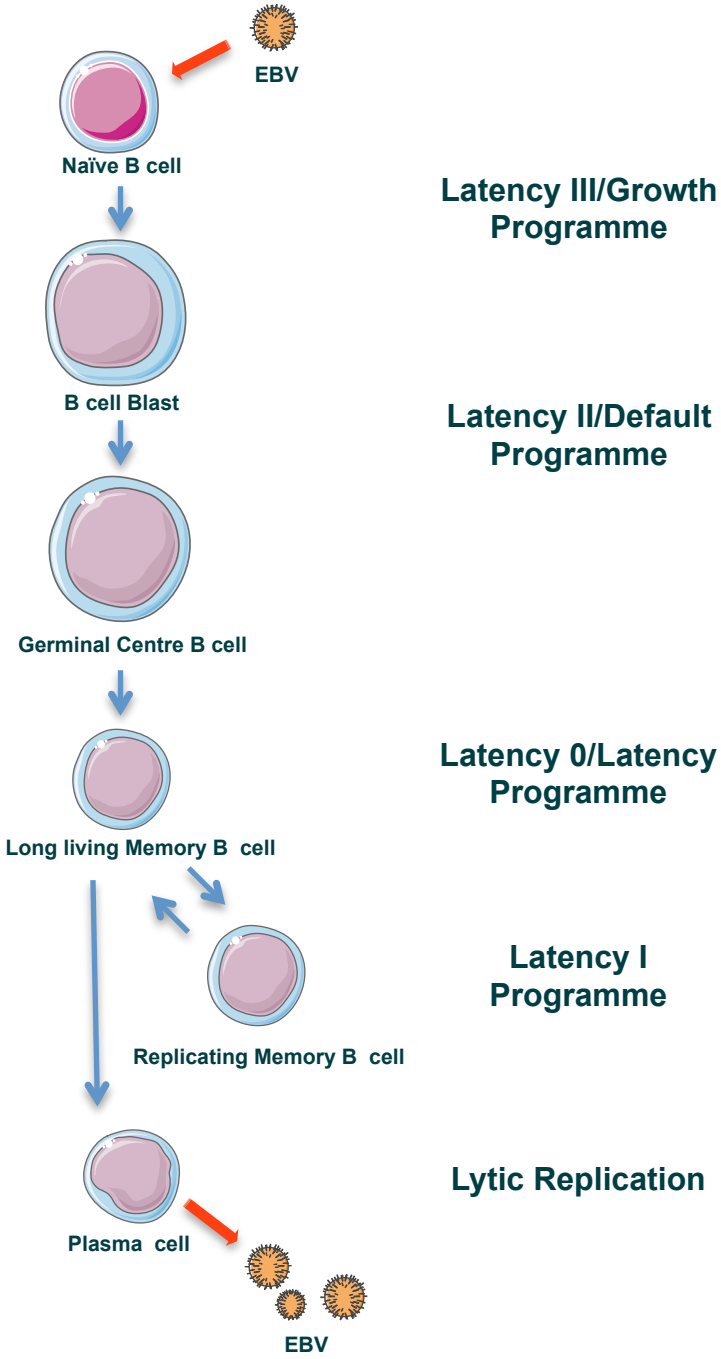


Figure 1.4 EBV life cycle in B cells.
Maturation pathway of B lymphocytes and the associated EBV life cycle programs are shown.

In contrast to the EBNA proteins, LMPs are transmembrane proteins and localise mainly in the outer lipid bilayer and lipid rafts (Higuchi et al., 2001). LMP1 is an analogue of CD40 protein, that binds to T cells through CD154 receptor, a process essential for B cell activation and survival. Unlike CD40, however, LMP1 is constitutively active and does not require CD154 binding, allowing the infected B cells to avoid apoptosis. LMP1 was also shown to be crucial for efficient viral release during the lytic cycle (Ahsan et al., 2005; D'Souza et al., 2000; Uchida et al., 1999). LMP2 proteins, on the other hand, are responsible for preventing reactivation of EBV. Antigen binding to the B cell receptor can result in a super activation of the phospholipase C pathway, ultimately triggering lytic cycle. Nonetheless, the LMP2 intracellular domain mimics that of the inactive B cell receptor and binds Lyn, Fyn and Syk protein kinases. In this bound form, the phosphorylation ability of these proteins is reduced, causing a tonic signal for survival but not a full activation of phospholipase C (Cohen, 2006; Miller et al., 1995; Thorley-Lawson, 2001).

1.2.4 EBV lytic replication.

Resting memory B cells that undergo terminal differentiation into plasma cells can cause reactivation of the lytic cycle, which will result in the production high numbers of new virion particles with eventual cell death. Conveniently, plasma cells preferentially migrate to the epithelial crypts to combat incoming infection by antibody secretion. This property of plasma cells provides a mechanism through which EBV can set a high viral load in the mucosa of the mouth, allowing the infection to spread throughout the population (Brandtzaeg et al., 1999; Laichalk and Thorley-Lawson, 2005).

As EBV switches to the lytic cycle, a cascade of events is initiated, that consist of three distinct stages: immediate early, early and late phases of protein expression. The main current model used for studying EBV reactivation *in vitro* utilises Burkitt's lymphoma derived cell lines (like Akata). Activating the B cell receptor (BCR) with such agents as IgG immunoglobulins *in vitro*, mimmics viral reactivation in plasma cells *in vivo* (Takada and Ono, 1989). Stimulation of the BCR in a functioning protein synthesis environment, was shown to result in upregulation of the two immediate early viral genes *BZLF1* and *BRLF1,* which encode the transcription factors, Zta and Rta (Flemington et al., 1991). These proteins modulate the expression of cellular and viral lytic genes by either directly binding to specific recognition sequences on the DNA or through other accessory proteins. Both transcription factors can initiate the lytic cycle since they can auto activate their own promoters known as Rp and Zp, and upregulate each other (Adamson et al., 2000; Hsu et al., 2005; Liu and Speck, 2003; Ragoczy et al., 1998). However, *in vitro* Luciferase assays have shown that only Zp (but not Rp) is directly upregulated by BCR activation. This suggests that Zta protein expression is a primary event, which is subsequently followed by the activation of the Rp (Amon et al., 2004). Binding sites of cellular transcription factors including myocyte enhancer factor-2 (MEF-2) and X-box binding protein 1 (XBP-1) have been identified in the *BZLF1* promoter region, and were shown to be essential for efficient transcription from Zp (McDonald et al., 2010). After Zta and Rta are expressed, the two proteins can synergise to alter cellular gene expression and cause G0/G1 to S cell cycle transition in B cells (Cox et al., 1990; Guo et al., 2010b). G0/G1 cell cycle arrest through inhibition of c-myc and activation of p21 and p27 is also attributed to Zta (Rodriguez et al., 2001). In addition to transcription control, Zta has the ability to bind to the origin of lytic replica-

tion (oriLyt) and trigger DNA synthesis required for new virions (Gao et al., 1998; Schepers et al., 1993a). Furthermore, Zta facilitates the switch from the viral latent phase by inhibiting the Cp promoter that drives the expression of the EBNA proteins (Sinclair et al., 1992). Nevertheless, both Zta and Rta are essential for the efficient progression of the lytic cycle (Feederle et al., 2000).

During the early lytic phase, with the help of Rta and Zta, EBV expresses a wide range of various genes. One group includes proteins that replicate the viral genome, allowing EBV to be independent of the cellular DNA polymerase. These are helicase-primase BBLF2/3, helicase BBLF4, primase BSLF1, DNA polymerase processivity factor BMRF1, single-stranded DNA binding factor BALF2 and DNA polymerase BALF5. Through an elaborate network of interaction with each other, Zta and DNA, the complex assembles at the oriLyt and initiates DNA synthesis. BALF5 and BMRF1 then carry on to replicate the EBV genome by rolling circle, creating long, concatameric DNA molecules, that are then cleaved at the terminal repeats and eventually packaged into virions [Figure 1.2] (Cohen, 2006). BMRF1 is also a transcription factor and together with another early lytic protein BRRF1, helps Zta and Rta with viral and host gene modulation (Holley-Guthrie et al., 2005; Hong et al., 2004). Another group of proteins in the early lytic cycle possesses antiapoptotic capabilities. BHRF1 and BALF1 are both homologues of cellular BCL-2 (B cell lymphoma 2) that is a proto-oncogene (Henderson et al., 1993; Marshall et al., 1999). BCL-2 inhibits BAX and BAK proteins that are found upstream of the caspase mediated apoptosis pathway (Youle and Strasser, 2008). Therefore BHRF1 and BALF1 can promote B cell survival needed in the highly immunogenic

lytic cells. The only EBV encoded protein kinase, BGLF4, is also an early lytic protein, whose function is discussed later on (Cohen, 2006; Kenney and Israle, 2005).

Late lytic proteins are classified as products expressed after viral DNA replication. Unlike early genes, they do not have Zta and Rta binding sites in their promoter region. Late proteins comprise all of the capsid proteins and surface glycoproteins that assemble the final virion particles. In addition to that, a cytokine homologue BCLF1, that shares almost 80% of its sequence with interleukin-10 (IL-10), is also expressed. Like its cellular counterpart, BCLF1 can stop the CTL response and stimulate B cell growth. It has been suggested that the secretion of BCLF1 from lytic cells can support the growth of the surrounding latently infected B cells (Hsu et al., 1990; Kenney and Israle, 2005; Salek-Ardakani et al., 2002).

1.3 EBV associated diseases.

Unlike other herpesviruses, most EBV associated diseases are linked to the latent phase rather than lytic viral replication. It is widely believed that causing a disorder is not a strategy actively pursued by EBV, as so much of the viral effort is dedicated to immune system avoidance, and persistence in the host. Therefore, some additional molecular aberrations must occur in the infected cells in order for a disease phenotype to develop. EBV related malignancies have been linked to different latency programs, with concomitant viral expression patterns (Thorley-Lawson, 2005).

The main affliction connected to the latency I program of EBV is Burkitt's lymphoma. BL is a B cell malignant tumour identified by over-expression of the oncogene

c-myc, that is a transcription factor involved, amongst other things, in apoptosis and cell proliferation. A translocation event of the c-myc ORF on chromosome 8 to highly active immunoglobulin promoter regions on either chromosome 2, 14 or 22, is thought to be a main driving event towards BL. What part EBV plays in the establishment of BL is not yet fully understood, but the extremely high rate of association of the virus with BL link them together. One possibility of EBV's involvement in BL, is the potential prevention of apoptosis and increased survival conferred by the viral EBER transcripts, that would allow the tumorigenic events to accumulate in the cells. On the other hand, EBV is thought to have the potential to cause chromosomal instability, that would lead to the c-myc translocation (Gruhne et al., 2009; Young and Rickinson, 2004).

Two major latency II associated diseases are Hodgkin's lymphoma and nasopharyngeal carcinoma. HL is a lymph node cancer thought to arise from overreaction of the immune system to the proliferation of malignant germinal centre B lymphocytes, called Hodgkin/Reed Sternberg cells. Up to 99% of the neoplasm volume consists of high numbers of various immune response cells like T lymphocytes, neutrophils, plasma cells, granulocytes, etc. NPC on the other hand, is an epithelial cancer most common is southeast Asia. Both HL and NPC use the growth promoting functions of LMP proteins discussed earlier (Thorley-Lawson, 2005; Young and Rickinson, 2004).

Latency III on the other hand, is related to EBV disorders that are found mostly in immunocompromised individuals. These include X-linked lymphoproliferative syndrome (XLP), post-transplant lymphoproliferative disorder (PTLD) and acquired immunodeficiency syndrome (AIDS) associated lymphomas. Without the appropriate T

cell response, B cells infected with EBV in the latency III program are not eliminated and the growth signals conferred by latency III genes are not restricted. Thus, B cells infected with EBV, in individuals with deficient immunity, are prone to lymphoproliferation (Kutok and Wang, 2006; Macsween and Crawford, 2003).

Oral hairy leukoplakia is also found in immunocompromised patients, but unlike most EBV diseases is associated with active lytic replication of the virus. OHL is a benign cell lesion found in epithelium of the mouth. It is not clear if the lytic cycle is reactivated in persistently infected epithelial cells due to decreased immune response or if the epithelium becomes more susceptible to the B cells amplification of EBV during immune suppression. Nonetheless, because OHL is linked to lytic replication, the lesions can be treated with acyclovir, which blocks the DNA synthesis in infected cells (Kutok and Wang, 2006; Macsween and Crawford, 2003).

1.4 Viral protein kinase

Viral Protein Kinase (VPK) is a early lytic protein encoded by the *BGLF4* gene. VPK is a serine/threonine kinase, 429 amino acids in length, that was discovered by homologous comparison (amino acid alignment) to the UL13 of HSV which is a conserved kinase throughout many herpesviruses. Other homologues of VPK include UL97 of human cytomegalovirus, gene 47 of varicella-zoster virus, 15R of human herpesvirus 6 and ORF36 of human herpesvirus-8 (Gershburg and Pagano, 2008). VPK was shown to be capable of autophosphorylation using ATP as well as GTP as substrate. Lysine 102 is a crucial residue for phosphorylation and the mutation of this site renders VPK kinase dead (Kato et al., 2003). VPK is mainly found in the nucleus with only a small amount

present in the cytoplasm. The nuclear localisation signal (NLS) of VPK is located within the 55aa of the C-terminus of the protein, which is different from the NLS of other herpesvirus kinases [Figure 1.5]. VPK is an early lytic gene whose expression rises during the progression of the lytic cycle and the protein co-localises in the viral replication compartments in the nucleus. However, VPK is also found in the tegument of the EBV virions from which it can detach through phosphorylation (Asai et al., 2006; Gershburg et al., 2004; Wang et al., 2005). VPK can phosphorylate numerous viral and host proteins (Zhu et al., 2009). The full range of potential targets is still unknown, and the studied interactions show involvement in diverse molecular processes that make it hard to identify a clear role for VPK. One of the early studies on VPK suggested that it mimicked the action of cyclin dependent kinase-1 (CDK1) by phosphorylating EBNA-LP and EF-1δ at the same residues. EF-1δ is an elongation factor required for the translation of cellular proteins. The ability of VPK to phosphorylate this factor suggest an involvement in protein synthesis in the cells (Kato et al., 2001; Kato et al., 2003; Kawaguchi et al., 2003). Moreover, both VPK and CDK1 can phosphorylate the viral DNA polymerase processivity factor BMRF1. This phosphorylation by VPK is thought to help the recruitment of Zta to the oriLyt ensuring efficient lytic replication (Chen et al., 2000; Yang et al., 2008). Another indication of CDK1 mimicry is the creation by VPK of mitosis-like conditions in the nucleus. Specifically, VPK can induce the condensation of the nuclear DNA by phosphorylating and activating topoisomerase II and condensin, which is thought to be useful for EBV as it creates more space for viral DNA replication (Lee et al., 2007). Additionally, VPK can also induce nuclear lamina disassembly by phosphorylating Lamin A protein essential for nuclear integrity. This is thought to help virions to egress from the nucleus, and is further supported by the fact that VPK can

control the disassembly of microtubules, another cytoskeletal component (Chen et al., 2010; Lee et al., 2008). Nevertheless, VPK does not fully mimic CDK1. A study aimed at finding VPK substrates using a protein array phosphorylation assay, identified a range of latent and lytic proteins, some of which were not CDK1 targets (Zhu et al., 2009). VPK was also shown to be involved in immune suppression by inhibiting interferon regulatory factor 3 (IRF3). In the presence of VPK, IRF3 has a diminished ability to bind to DNA and upregulate interferon production. The inhibition is thought to be through phosphorylation of Serines 123 and 173, and Threonine 180 of IRF3 (Wang et al., 2009). Furthermore, VPK involvement in the DNA damage response is highlighted by direct phosphorylation of the histone acetyltransferase TIP60 (Li et al., 2011). Zta is another protein known to be targeted by VPK. Zta is phosphorylated at Serine 209 which is also a non-CDK1 site but rather similar to a casein kinase 2 (CK2) site. After phosphorylation VPK and Zta can form a stable complex which might lead to their co-localization. In their study, Asai et al. also observed some down-regulation of Zta trans-activation activity of its own Zp promoter when VPK was present. They suggested that this might be a control mechanism to decrease Zta levels in later stages of lytic cycle (Asai et al., 2006; Asai et al., 2009).

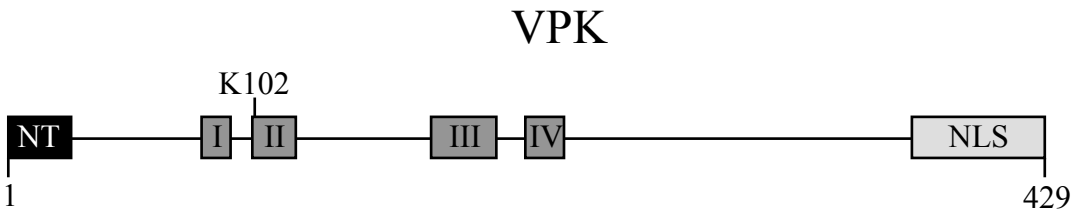


Figure 1.5 Graphical representation of VPK's primary structure.
The catalytic Lysine 102 (K102) as well as the first 26 residues (NT) required for (auto)phosphorylation are shown. The domains homologous to other Herpesvirus protein kinases are indicated by roman numerals, with number II predictively being the ATP binding site; the terminal 55aa responsible for nuclear localisation are marked as NLS. Based on Chen et al. (2000) and Gershburg et al. (2004) studies.

1.5 Zta

Zta, also known as Z, Zebra or EB1 is an immediate-early lytic protein that is encoded by the *BZLF1* gene of EBV. Zta is a transactivator that can also act as a transcriptional repressor or DNA replication factor. Among the many functions of Zta are lytic cycle progression, initiation of viral DNA synthesis, cell cycle regulation, immune response modulation and inhibition of apoptosis (Sinclair, 2003).

1.5.1 Zta protein structure.

Zta is a 245 amino acid in length, 35kDa protein that belongs to the basic leucine zipper (bZIP) family of transcription factors which include cellular homologues like c-jun and c-fos. bZIP proteins have a characteristic basic region responsible for DNA contact and a leucine zipper (ZIP) region involved in dimerisation. The basic domain of Zta has a high homology with c-fos while the zipper is somewhat atypical. Both of these motifs are found in the carboxy terminal region of the protein [Figure 1.6] (Farrell et al., 1989). Investigations using electrophoretic mobility shift assays (EMSAs) have shown that the C-terminal 134-245aa Zta fragment alone can bind DNA (Farrell et al., 1989). The basic domain, located roughly between the residues 175 and 195, is composed of two distinct parts termed basic region A (BRA) and basic region B (BRB). Mutagenesis experiments of BRA and BRB have shown that these regions are essential for DNA binding, as well as, nuclear localisation of Zta (Mikaelian et al., 1993; Packham et al., 1990).

The zipper region of Zta is located approximately between residues 195 and 221 [Figure 1.6], and is crucial for dimerisation, which in turn is a prerequisite for DNA

binding (Hicks et al., 2003). Early studies proposed that the dimerisation of Zta is dependent on a coiled coil helix formation. However, unlike other bZIP proteins, Zta's bZIP domain does not contain heptad leucine repeats but rather repeats of hydrophobic amino acids. This in part explains the inability of Zta to efficiently heterodimerise with other bZIP proteins like c-jun and c-fos. Mutational evaluation of the zipper region demonstrated that the amino acids on the inside of the predicted helix are necessary for dimerisation, whereas the substitution of the outside residues does not elicit an effect (Flemington and Speck, 1990). Indeed, crystallographic analysis of a dimerised 175 to 236 amino acid portion of Zta bound to DNA, confirmed that both basic and zipper regions form a helix. The basic part of the protein fits in the major groove of the DNA, but unlike other bZIP transcriptions factors, binds with a higher affinity. This allows Zta to interact with a larger variety of DNA sequences in comparison to other bZIP proteins. In addition, Zta's zipper region does form a coiled coil but is shorter than that of c-jun or c-fos. A proline residue at position 223, allows the short C-terminal helix to fold back onto itself and interact with the coiled coil structure, contributing to the stability of the homodimer (Petosa et al., 2006). Further biophysical and biochemical investigations questioned the importance of the carboxy terminus of Zta. Schelcher et al. (2007) generated short peptides of the bZIP domain (196-231aa of Zta) or bZIP with 10 extra adjacent C-terminal residues (196-221aa of Zta), and subjected them to analytical centrifugation and circular dichroism spectroscopy. The addition of 10 CT amino acids considerably increased the dimer and helical structure formation in the studied fragments. In addition, the group also produced a series of carboxy terminal truncation mutants of Zta. Cross-linking experiments showed that deletions past residue 230 (1-227aa fragments or shorter), prevents Zta from forming protein dimers. While EMSA and reverse transcrip-

tion quantitative PCR (qPCR) demonstrated that Zta must be at least 230 residues long to interact with DNA *in vitro* or transactivate the *BMRF1* gene *in vivo* (Schelcher et al., 2007). Interestingly, in a separate study, truncating just the three CT residues (243-245aa) proved to be sufficient for the inhibition of viral genome replication *in vivo* and Zta binding to p53-binding protein 1(53BP1) *in vitro* (Bailey et al., 2009). Thus, Zta is a unique member of bZIP family of proteins which has evolved an uncommon helix motif and carboxy terminal region, allowing it to predominantly form homodimers and to bind a wider range of DNA and protein targets.

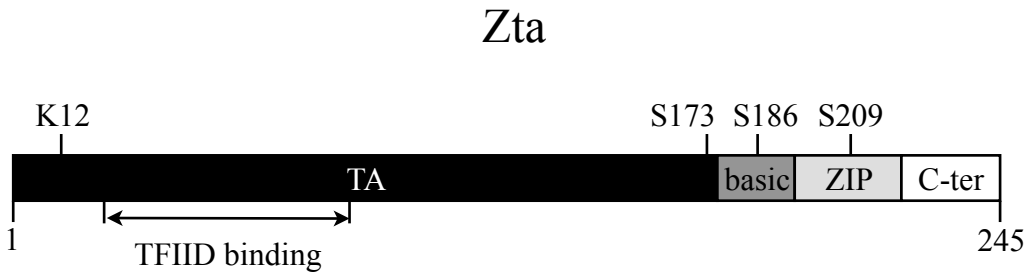


Figure 1.6 Graphical representation of Zta's primary structure.
The key residues are shown: K12 - Lysine 12 at which sumoylation occurs and S173, S186 and S209 - Serines 173, 186 and 209 at which phosphorylation by CK2, PKC and VPK occurs. The main known domains are also shown: TA - transactivation domain; DNA - basic DNA contact region; bZIP - basic zipper domain; C-ter - Carboxy terminal region. TFIID binding region associated with highest transactivation loss upon mutation is also shown.

The rest of the (amine adjacent) Zta protein is a much less structured region than the bZIP domain and has not yet been crystallised. Even though the first 174 amino acids are not involved in the DNA binding ability of Zta, the amine terminus is known to

be important for the transcription activity of the protein and is therefore called the transactivation domain (TA) (Packham et al., 1990). A hybrid protein consisting of the first 167 residues of Zta joined to the dimerisation and DNA binding regions of Gal4 can activate a promoter with Gal4 binding sites *in vitro*. In the same study, a sequential mutational analysis of the TA region revealed that several elements are responsible for the transactivation function of Zta. Mutating the protein past amino acid 153 and up to residue 167 abolishes its DNA binding ability, possibly due to close proximity to the bZIP region. The highest loss in the transcriptional activity (80% decrease) of the Zta variants is associated with deletion of 27-53aa and 52-78aa regions. Removing other short TA sections however, also correlates with a decrease in transcription activation, albeit to a lesser extent (40% decrease) (Flemington et al., 1992). Point mutation experiments showed that various amino acid groups in the first 78 residues of Zta have cell type specific and functional differences. One group is the negatively charged residues, aspartic acid 28 and 35 together with glutamic acid 54 (D28 D25 E54); the other consists of aromatic amino acids, phenylalanine 22, 26 and 75 with tryptophan 49 and 74 (F22 F26 F75 W49 W74). The acidic D28, D25 and E54 residues are involved in the transcription of the *BMRF1* and *BRLF1* promoters as well as Zta dependent reactivation of the lytic cycle in both epithelial and B-cells. Whereas the hydrophobic F22, F26, F75, W49 and W74 residues are needed for Zta's transcription of a range of viral promoters in epithelial cells and interaction with CREB-binding protein (CBP) and TFIIA-TFIID transcription factors. Additionally, the acidic residues play a role in stabilising Zta, whereas mutating the aromatic amino acids cause a decrease in protein levels (Deng et al., 2001). As a result, it is thought that the diverse amino acid composition and the flexibility of

the TA domain allows Zta to bind a range of various targets and exhibit a plethora of effects.

1.5.2 Post-translational modifications of Zta.

Zta is post-translationally modified in several different ways. One type of PTM associated with Zta is the phosphorylation on a range of different residues. Point mutations of the protein, in conjunction with various biochemical techniques, have revealed several critical phospho-modified residues involved in the function of Zta. By employing *in vitro* kinase assays, Baumann et al. (1998) demonstrated that Protein kinase C (PKC) can phosphorylate Zta on Serine 186. This phosphorylation was also achieved *in vivo,* by induction with a PKC activator, 12-O-tetradecanoylphorbol-13-acetate. Additionally, EMSAs showed that the S186A mutant significantly reduces the ability of Zta to bind DNA *in vitro*. The same mutant did not activate *BHLF1* and Rp Luciferase promoter constructs. Interestingly, *in vitro* DNA interaction of Zta was enhanced by incubating the phosphorylated protein with cellular extracts. Therefore, S186 phosphorylation is required for efficient promoter activation and DNA binding, but is possibly dependent on the interaction with other cellular factors (Baumann et al., 1998). Furthermore, western blot analysis showed that a phosphomimmic mutation S186E, but not the phosphorylation incapable S186A, interferes with Zta's inhibition of c-myc, suggesting that the unphosphorylated S186 residue might also have the ability to recruit other proteins (Rodriguez et al., 2001). Another phosphorylation site on Zta is Serine 173. Kolman et al. (1993), employed a radiolabelling *in vitro* kinase assay to demonstrate that CK2 can phosphorylate Zta on this residue (Kolman et al., 1993). Further immunoblot, EMSA and quantitative PCR performed with S173A mutants revealed that Serine 173 is

crucial for the ability of Zta to induce lytic EBV replication, associate with DNA and transactivate late lytic genes (like *BFRF3* and *BLFR2*). Nevertheless, the S173A mutant does not completely abolish DNA interactions and has no effect on transcription of early lytic genes (like Rta or the viral replication machinery proteins) by Zta. This implies that Serine 173 phosphorylation might have a higher relevance in the later stages of the lytic cycle (El-Guindy et al., 2007). As mentioned before, Zta is also phosphorylated by VPK on Serine 209, which causes a decrease in auto-activation of the Zp promoter construct. In addition to that, diminishing *BZLF1* mRNA levels correlate with increasing VPK protein presence during the lytic cycle progression *in vivo* (Asai et al., 2009). El-Guindy et al. (2006) employed radiolabelling of Zta point mutants in combination with tryptic digestion and two dimensional gel electrophoresis to demonstrate that Zta can also be phosphorylated on Serine residues 6, 8 and 167, as well as Threonine residues 7, 14 and 159. However, the significance and role of these PTMs *in vivo* still remains to be appreciated (El-Guindy et al., 2006).

Zta can also be post-translationally modified by SUMO conjugation (Adamson and Kenney, 2001). SUMOs or Small Ubiquitin-like Modifiers are a family of proteins that are approximately 20% homologous to Ubiquitin, and consist of four family members. SUMO2 and 3 are very similar in function and amino acid sequence, and share about 50% homology with SUMO1. SUMO4 on the other hand is rarely observed *in vivo,* and its role still remains much less understood than the other three proteins. SUMO is covalently attached to Lysine amino acids in a range of target proteins and can results in various effects, including altered cellular localisation or modification of transcription ability. In addition, SUMO can block other Lysine PTMs and associated effects. In this

manner, by occupying a Lysine residue that can also be Ubiquitylated, SUMO can protect a protein from Ubiquitin-dependent proteasomal degradation (Hay, 2005). Zta is SUMOylated on Lysine 12. Mutating this residue abrogates higher molecular weight forms of Zta that react to SUMO antibodies. Introduction of Zta into cells can cause a decrease in SUMOylation of Promyelocytic Leukemia (PML) protein through competition. SUMO does not influence the localisation or stability of Zta in the cells. However, SUMO co-transfection with Zta in HeLa cells decreases the transcription of Rp and *BMRF1,* but not Zp promoters cloned into a Luciferase vector. Additionally, *BMRF1* promoter activation can be rescued by expressing Rta together with SUMO and Zta (Adamson, 2005; Adamson and Kenney, 2001). Moreover, mutating Lysine 12 and Phenylalanine 13 residues, is associated with severely decreased production of infectious virions, suggesting a significant role of Zta SUMOylation in the EBV's lifecycle (Deng et al., 2001).

1.5.3 Zta and DNA binding.

As a transcription factor, Zta can directly bind to the DNA and modulate gene expression. Zta response elements (ZREs) are seven nucleotide long sequences that mediate DNA binding with the protein and are found across many viral and cellular promoters (Flower et al., 2010). Karlsson et al. (2008) proposed that the ZREs could be grouped into three distinct classes: class I, class II and class III. Class I ZREs lack CpG motifs and are inherently independent of DNA methylation for Zta binding. Class II and class III ZREs on the other hand, do contain CpG dinucleotides and are affected by cytosine methylation. While class III ZREs can bind Zta only in the methylated form, class II can recruit Zta regardless of the methylation state (Karlsson et al., 2008). Nonetheless, an increasing amount of evidence suggests that Zta binds more efficiently and

therefore prefers methyl-ZREs. Recently, chromatin immunoprecipitation coupled with next generation sequencing (ChIP-seq) studies have shown that Zta interacts with *in vitro* methylated EBV DNA with a higher affinity than the unmethylated genome. Validation of several crucial lytic promoters with EMSA and Luciferase assays showed that Zta binds and activates ZREs that contain CpG motifs with greater efficiency when they are in a methylated state (Bergbauer et al., 2010). Kalla et al. (2012) confirmed these observations at the mRNA level by reverse transcription qPCR. Together, these findings explain the absence of virion production during primary EBV infection when the viral genome is unmethylated (Kalla et al., 2010). As latency progresses, the viral genome becomes methylated and stays so until the early lytic cycle, allowing Zta mediated reactivation (Woellmer et al., 2012). Another ChIP-seq study, however, revealed that during the transition from early to late lytic cycle, the fall in viral genome methylation correlates with reduced amounts of Zta bound to CpG ZREs, while methylation-independent ZREs exhibit higher Zta occupancy. This suggests that Zta's functional role might change with the progression of the lytic cycle (Ramasubramanyan et al., 2012a). Whether the demethylation of the viral genome during the later stages of the lytic phase is an active process is not yet known. Nevertheless, it is becoming evident that Zta's ability to differentially bind and activate three classes of ZREs is intimately linked to the control of EBV's life cycle.

1.5.4 Zta and the viral lytic replication.

At the oriLyt, Zta brings together the viral DNA polymerase complex and aids the *de novo* DNA synthesis. Zta is essential for initiation of the viral lytic replication and plays the role of the origin binding protein (Fixman et al., 1995). It is thought that a

DNA hairpin formation by the two ZREs found in the upstream essential element of the oriLyt, is needed for efficient Zta binding (Rennekamp et al., 2010). Zta also directly interacts with EBV's primase-helicase complex and viral polymerase processivity factor, linking oriLyt binding to initiation of EBV lytic replication (Gao et al., 1998; Zhang et al., 1996).

1.5.5 Transcriptional activation by Zta.

To numerous viral and cellular promoters that contain ZREs, Zta recruits transcription factors, thereby initiating mRNA synthesis. Among the most studied is the ability of Zta to assemble the TFIIA-TFIID transcription machinery on the DNA. Zta can directly bind to the TATA binding TFIID, and increase its promoter affinity. This protein-protein interaction is dependent on amino acids 25 to 86 of Zta (Ellwood et al., 1999; Lieberman and Berk, 1991). The mentioned ChIP-seq studies have also identified many viral promoters associated with Zta. Apart from validating Zta occupancy on the previously established Rp, Zp and early lytic promoters *in vivo*, a whole range of early and late EBV genes bound to Zta have been discovered. The evidence suggests that Zta has viral genome-wide effects during the lytic cycle (Bergbauer et al., 2010; Ramasubramanyan et al., 2012a). Zta can also induce transcription of cellular genes to create potentially favourable conditions for EBV lytic replication. Accordingly, Zta is known to upregulate the transcription of IL-10, a cytokine capable of suppressing both MHC class I and class II antigen presentation. Indeed, EMSAs showed that Zta can directly bind to the ZREs found in the IL-10 promoter. Mutating the TA domain or the basic region of Zta abolishes IL-10 transactivation (Mahot et al., 2003). On the other hand, Zta is also found in the promoter region of an EBV encoded IL-10 homologue, BCRF1, highlight-

ing the importance of this protein for the viral lytic phase (Ramasubramanyan et al., 2012a). Although the exact cell cycle conditions suitable for the lytic replication remain debated, Zta can arrest the cells in the G1 stage by activating a CDK1 inhibitor, p21. The upregulation of p21 is dependent on Zta directly binding to another bZIP transcription factor, CCAAT/Enhancer Binding Protein α (C/EBPα). The two proteins can then act together to transactivate p21, as well as C/EBPα and BZLF1 gene expression. However, Zta and C/EBPα do not seem to heterodimerise but rather interact with each other as homodimers (Wu et al., 2003; Wu et al., 2004).

1.5.6 Transcriptional repression by Zta.

The process of transcriptional repression of Zta by direct DNA binding is controversial and not well understood. By employing EMSAs, Sato et al. (1992) showed that Zta can bind to the c-jun promoter sequence *in vitro*. In addition to that, Chloramphenicol AcetylTransferase (CAT) assays revealed that when co-transfected with c-jun, Zta can inhibit activation of the collagenase and c-jun promoter constructs. Therefore, repression seems to be dependent on competition of Zta with c-jun for the promoter region occupancy (Sato et al., 1992). On the other hand, Zta can also down-regulate the class II major histocompatibility complex transactivator (CIITA) pIII promoter. Li et al. (2009) showed that Zta can bind to the transcription start site (TSS) proximal ZRE found in the pIII of *CIITA in vivo*. The DNA binding dependent repression is substantiated by the fact that mutating the proximal ZRE sequence, or the basic region of the transiently expressed Zta, abrogates the downregulation of the *CIITA* promoter construct. However, no further steps into investigating the exact mechanism of action of Zta mediated repression were taken (Li et al., 2009). Additionally, no known transcription

factor binding sites overlap with the ZRE in the pIII (Ghosh et al., 1999; van der Stoep et al., 2004; van der Stoep et al., 2002). Therefore it is unlikely that the competition model is relevant to the repression of the *CIITA* promoter by Zta.

1.6 Thesis aims.

To investigate the effects of VPK on Zta's post-translational modifications and the involvement of both proteins in the DNA damage response, particularly phosphorylation of H2AX.

To identify protein coding regions and post-translational modifications of Zta implicated in *CIITA* pIII repression and *BHLF1* promoter activation.

To determine which DNA elements in the *CIITA* promoter are required for Zta repression.

2. Materials and Methods.

2.1 Materials.

2.1.1 Plasmids.

(backbone) Plasmid Name	Purpose	Origin
pcDNA3	WB (western blotting), Luciferase assays, ChIP	Invitrogen
(pcDNA3) his-Zta	WB, Luciferase assays, ChIP	(Bailey et al., 2009)
(pcDNA3) his-Zta K12R	WB, Luciferase assays, ChIP	Generated myself
(pcDNA3) his-Zta S209A	WB, Luciferase assays	Generated myself
(pcDNA3) his-Zta S209D	WB, Luciferase assays	Generated myself
(pcDNA3) his-SUMO1-Zta	WB, Luciferase assays, ChIP	Generated myself
(pcDNA3) his-SUMO2-Zta	WB, Luciferase assays, ChIP	Generated myself
(pcDNA3) his-Zta ΔTA	WB, Luciferase assays	Generated myself
(pcDNA3) his-Zta 243ter	WB, Luciferase assays	Provided by the lab
(pcDNA3) his-Zta AAA	WB, Luciferase assays	Provided by the lab
(pcDNA3) his-Zta 199ter	WB, Luciferase assays	Generated myself
(pcDNA3) his-Zta 134ter	WB, Luciferase assays	Generated myself
(pcDNA3) his-Zta 166ter	WB, Luciferase assays	Generated myself
(pcDNA3) his-Zta 26-199ter	WB, Luciferase assays	Generated myself
(pcDNA3) his-Zta 56-199ter	WB, Luciferase assays	Generated myself
(pcDNA3) his-Zta 81-199ter	WB, Luciferase assays	Generated myself
(pcDNA3) VPK	WB, Flag pulldowns	Provided by the lab
pBabe	WB, Flag pulldowns	(Morgenstern and Land, 1990)
(pBabe) Zta	WB, Flag pulldowns	(Bailey et al., 2009)
(pBabe) Zta K12R	WB, Flag pulldowns	Generated myself
(pBabe) Zta S209A	WB, Flag pulldowns	Generated myself

(backbone) Plasmid Name	Purpose	Origin
(pBabe) Zta S209D	WB, Flag pulldowns	Generated myself
pCpGL	Luciferase assays	(Klug and Rehli, 2006)
(pCpGL) BHLF1	Luciferase assays	(Bergbauer et al., 2010)
pGL3 control	Luciferase assays	Promega
pGL3 enhancer	Luciferase assays	Promega
(pGL3 enh.) CIITA	Luciferase assays	Generated myself
(pGL3 enh.) CIITA null ZRE	Luciferase assays	Generated myself
(pGL3 enh.) CIITA ZRE 3/4	Luciferase assays	Generated myself
(pGL3 enh.) CIITA -199+44	Luciferase assays	Generated myself
(pGL3 enh.) CIITA -199+52	Luciferase assays	Generated myself
(pGL3 enh.) CIITA -214+44	Luciferase assays	Generated myself
(pGL3 enh.) CIITA mut H	Luciferase assays	Generated myself
(pGL3 enh.) CIITA p50 mut	Luciferase assays	Generated myself
(pGL3 enh.) CIITA downstream mut	Luciferase assays	Generated myself
(pGL3 enh.) CIITA 1-40 mut	Luciferase assays	Generated myself
(pGL3 enh.) CIITA 41-80 mut	Luciferase assays	Generated myself
(pGL3 enh.) CIITA 81-121 mut	Luciferase assays	Generated myself
(pGL3 enh.) CIITA 121-160 mut	Luciferase assays	Generated myself
(pGL3 enh.) CIITA 161-214 mut	Luciferase assays	Generated myself

Table 2.1 List of plasmids used in the experiments.

2.1.2 Oligonucleotides

Primer name	Purpose	Sequence
K12R_F	Generating his-Zta K12R and Zta K12R mutants by site-directed mutagenesis	CTCGACTTCTGAAGATGTAAG ATTTACACCTGACCCATACC
K12R_R	Generating his-Zta K12R and Zta K12R mutants by site-directed mutagenesis	GGTATGGGTCAGGTGTAAATC TTACATCTTCAGAAGTCGAG
S209A_F	Generating his-Zta S209A and Zta S209A mutants by site-directed mutagenesis	GGCTGCTGCCAAATCAGCTG AAAATGACAGGCTGCGCC
S209A_R	Generating his-Zta S209A and Zta S209A mutants by site-directed mutagenesis	GGCGCAGCCTGTCATTTTCAG CTGATTTGGCAGCAGCC
S209D_F	Generating his-Zta S209D and Zta S209D mutants by site-directed mutagenesis	GGCTGCTGCCAAATCAGATG AAAATGACAGGCTGCGCC
S209D_R	Generating his-Zta S209D and Zta S209D mutants by site-directed mutagenesis	GGCGCAGCCTGTCATTTTCAT CTGATTTGGCAGCAGCC
his-Zta_F_ normal	Generating his-Zta 243ter, his-Zta AAA and his-Zta 199ter mutants for subcloning into pcDNA3 vector.	CTGCACACCGGGGATCCATGC ATCATCATCATCATCATATGAT GGACCCAAACTCGACTTCT
his-Zta_R_ normal	Generating his-Zta 199ter mutant for subcloning into pcDNA3 vector.	CTGCACACCGGGGAATTCTTA GAAATTTAAGAGATCCTCGTG TAA
his-Zta_R_ 243ter	Generating his-Zta 243ter mutant for subcloning into pcDNA3 vector.	CTGCACACCGGGGAATTCTTA GAAATTTAACTAGAGATCCTC GTG

Primer name	Purpose	Sequence
his-Zta_R_ AAA	Generating his-Zta AAA mutant for subcloning into pcDNA3 vector.	CTGCACACCGGGGAATTCTTA AGCAGCAGCGAGATCCTCGT G
CIITA null_F	Generating CIITA null plasmid by site-directed mutagenesis	TGAAGGTTCCCCCAACAGAC TTTCTCCCTTCTTTCTGTCTTC ACCAAATTCAGTC
CIITA null_R	Generating CIITA null plasmid by site-directed mutagenesis	GACTGAATTTGGTGAAGACA GAAAGAAGGGAGAAAGTCTG TTGGGGGAACCTTCA
CIITA B3/4_F	Generating CIITA B3/4 plasmid by site-directed mutagenesis	AAGGTTCCCCCAACAGACTT TCTGACACACTTTCTGTCTTC ACCAAATTCAG
CIITA B3/4_R	Generating CIITA B3/4 plasmid by site-directed mutagenesis	CTGAATTTGGTGAAGACAGA AAGTGTGTCAGAAAGTCTGT TGGGGGAACCTT
CIITA +44	Generating CIITA -199+44 and CIITA -214+44 promoters for subcloning	GCCCCAAGCTTCCCAGCTCA GAAGCACAC
CIITA +52	Generating CIITA -199+52 and CIITA -214+52 promoters for subcloning	GCCGGAAGCTTTTCGGATGCC CAG
CIITA -199	Generating CIITA -199+52 and CIITA -199+44 promoters for subcloning	GCCGGTACCAAATTCAGTCCA CAGTAAGGAAGTG
CIITA -214	Generating CIITA -214+52 and CIITA -214+44 promoters for subcloning	GCCGGTACCCTTTCTGTCTTC ACCAAATTCAGTCC
Rp3_F	qPCR	GGCTGACATGGATTACTGGTC
Rp3_R	qPCR	TGATGCAGAGTCGCCTAATG

Primer name	Purpose	Sequence
oriLyt flank_F	qPCR	CCGCATGTCCAACCACCACG
oriLyt flank_R	qPCR	ATGCTACCTAGGCCTGCGTCC
Rp_F (+biotin)	DNA affinity chromatography	ATATTGCGATTGCCCGCCCTATGCCAATGGCTCATAA + biotin
Rp_R	DNA affinity chromatography	TTATGAGCCATTGGCATAGGGCGGGCAATCGCAATAT
Rp mut_F (+biotin)	DNA affinity chromatography	ATATTGCGATTGCCCGCCCTATGCCAAGACTGCATAA + biotin
Rp mut_R	DNA affinity chromatography	TTATGCAGTCTTGGCATAGGGCGGGCAATCGCAATAT

Table 2.2 List of ordered oligonucleotides.

2.1.3 Antibodies.

Antibody (order)	Purpose	Species, clonality	Origin
anti-Flag M2 (primary)	WB	Mouse, monoclonal	Sigma
anti-γH2AX (primary)	WB	Mouse, monoclonal	Upstate
anti-β actin (primary)	WB	Rabbit, polyclonal	Sigma
BZ1 (primary)	WB	Mouse, monoclonal	Young el al. (1991)
DO1 (primary)	WB	Mouse, monoclonal	Santa Cruz Biotech
anti-Rta (primary)	WB	Mouse, monoclonal	Argene
anti-Cdk2 (primary)	WB	Mouse, monoclonal	Santa Cruz Biotech
ScZ (primary)	WB, ChIP	Goat, polyclonal	Santa Cruz Biotech
HRP-linked anti-mouse IgG (secondary)	WB	Horse	GE Healthcare
HRP-linked anti-rabbit IgG (secondary)	WB	Goat	GE Healthcare

Antibody (order)	Purpose	Species, clonality	Origin
HRP-linked anti-goat IgG (secondary)	WB	Rabbit	DAKO
IRDye 680CW anti-mouse IgG (secondary)	WB	Goat	Li-Cor
IRDye 800CW anti-rabbit IgG (secondary)	WB	Goat	Li-Cor
IRDye 680CW anti-goat IgG (secondary)	WB	Donkey	Li-Cor

Table 2.3 List of antibodies used for western blotting and chromatin immunoprecipitation.

2.1.4 Purchased reagents and materials.

Reagents and materials	Purpose	Supplier
Luria-Bertani (LB) agar powder	Bacterial cell culture (after re-suspension)	Invitrogen
Luria-Bertani (LB) broth powder	Bacterial cell culture (after re-suspension)	Invitrogen
Penicillin	Plasmid amplification	GIBCO
Zeocin	Plasmid amplification	Invitrogen
Low melting point agarose	Plasmid amplification	Sigma
10x Pfx enhancer solution	PCR	Invitrogen
10x Pfx amplification buffer	PCR	Invitrogen
Platinum Pfx DNA polymerase	PCR	Invitrogen
dNTP mix (10mM each)	PCR	New England BioLabs
DpnI restriction enzyme (RE)	PCR	New England BioLabs

Reagents and materials	Purpose	Supplier
KpnI high fidelity RE	Subcloning	New England BioLabs
HindIII high fidelity RE	Subcloning	New England BioLabs
EcoRI high fidelity RE	Subcloning	New England BioLabs
BamHI RE	Subcloning	Roche
RE buffer 4	PCR, Subcloning	New England BioLabs
T4 DNA ligase	Subcloning	Invitrogen
5x T4 ligase buffer	Subcloning	Invitrogen
DMSO	Cell Culture	GIBCO
DMEM	Cell Culture	GIBCO
RPMI 1640	Cell Culture	GIBCO
Foetal calf serum (FCS)	Cell Culture	Sigma
Penicillin, Streptomycin, L-Glutamine (PSG)	Cell Culture	GIBCO
Antimycotic, antibiotic (AA)	Cell Culture	GIBCO
Hygromycin B	Cell culture	Invitrogen
L-glutamine	Cell Culture	GIBCO
DPBS	Cell Culture, Transfections, Benzonase lysis	GIBCO
Effectene	Transfections	Qiagen
Enhancer	Transfections	Qiagen
Buffer EC	Transfections	Qiagen
6-well plate (3.5cm diameter)	Transfections	NUNC
25cm² small flask	Transfections	NUNC
75cm² medium flask	Transfections	NUNC
4mm gap electroporation cuvettes	Transfections	VWR
NuPAGE Bis-Tris Precast Gels	SDS-PAGE	Invitrogen

Reagents and materials	Purpose	Supplier
NuPAGE SDS MOPS running buffer	SDS-PAGE	Invitrogen
Odyssey blocking buffer	WB	Li-Cor
PBS (100 tablets)	WB	Oxoid
Tween 20	WB	Fisher BioReagents
2x protein sample buffer (2xPSB)	WB	Sigma
SeeBlue Pre-stained markers	WB	Invitrogen
Luminol	WB	Sigma
P-Coumaric acid	WB	Sigma
Hydrogen peroxide 30%	WB	Sigma
Complete™ EDTA-free protease inhibitor cocktail	Flag pulldowns, ChIP	Roche
Benzonase nuclease	Flag pulldowns	Novagen
EZview Red anti-Flag M2 Affinity Gel	Flag pulldowns	Sigma
Luciferase assay substrate	Luciferase assays	Promega
Luciferase assay buffer	Luciferase assays	Promega
5xPassive Lysis Buffer (PLB)	Luciferase assays	Promega
Protein assay	Luciferase assay quantification	Bio-Rad
Protein A-Sepharose 4B, Fast Flow	ChIP	Sigma
Protein G-Sepharose, Fast Flow	ChIP	Sigma
Salmon sperm DNA (10mg/ml)	ChIP	Invitrogen

Reagents and materials	Purpose	Supplier
Triton X-100	ChIP	Sigma
Proteinase K (10mg/ml)	ChIP	Sigma
GoTaq qPCR master mix	ChIP	Promega
M-270 Streptavidin Dyna-beads	DNA affinity chromatog-raphy	Invitrogen
2x B&W buffer	DNA affinity chromatog-raphy	Invitrogen
5x gel shift binding buffer	DNA affinity chromatog-raphy	Promega

Table 2.4 List of reagents and materials purchased from various suppliers.

2.1.5 Solutions.

Solution	Purpose	Composition
Buffer I	Generating competent cells	10mM $CaCl_2$, 50mM $MnCl_2$•$4H_2O$, 30mM KOAc, 100mM RbCl, 15% v/v glycerol; pH 5.8
Buffer II	Generating competent cells	10mM RbCl, 10mM MOPS, 75mM $CaCl_2$, 15% v/v glycerol; pH 6.8
ECL solution I	WB	10mM Tris HCl pH8.5, 0.4mM p-Coumaric acid, 2.5mM Luminol
ECL solution II	WB	10mM Tris HCl pH8.5, 0.02% Hydrogen Peroxide

Solution	Purpose	Composition
PPI (50x)	Benzonase lysis, Flag pulldowns	50mM NaF, 10mM Na_3VO_4, 50mM EGTA, 500mM $Na_4P_2O_7$, 100mM β glycerophosphate
Benzonase lysis buffer	Benzonase lysis, Flag pulldowns	50mM Tris (pH7.5), 2mM $MgCl_2$, 50mM NaCl, 1mM NEM, 0.5% NP40, EDTA-free protease inhibitors, 1xPPI
Cell lysis buffer	ChIP	85mM KCl, 0.5% NP-40, 5mM PIPES pH8, 1mM PMSF, EDTA-free protease inhibitor cocktail
SDS lysis buffer	ChIP	1% SDS, 10mM EDTA, 50mM Tris pH8, 1mM PMSF, EDTA-free protease inhibitor cocktail
ChIP dilution buffer	ChIP	0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris pH 8, 167mM NaCl, 1mM PMSF, EDTA-free protease inhibitor cocktail
Low salt buffer	ChIP	0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8, 150mM NaCl, 1mM PMSF, EDTA-free protease inhibitor cocktail

Solution	Purpose	Composition
High salt buffer	ChIP	0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8, 500mM NaCl, 1mM PMSF, EDTA-free protease inhibitor cocktail
LiCl buffer	ChIP	250mM LiCl, 1% NP-40, 1% Na-deoxycholate, 1mM EDTA, 10mM Tris pH8, 1mM PMSF, EDTA-free protease inhibitors
TE buffer	ChIP	10mM Tris, 1mM EDTA, 1mM PMSF, EDTA-free protease inhibitor cocktail
Elution buffer	ChIP	10mM Tris, 5mM EDTA, 1mM PMSF, 1% SDS, EDTA-free protease inhibitor cocktail
Binding buffer	DNA affinity chromatography	1.5x promega gel shift binding buffer, 6mM DTT, 72ng/ml poly(dI-dC),
D150	DNA affinity chromatography	20mM HEPES pH 7.9, 20% Glycerol, 0.2mM EDTA, 0.05% NP40, 150mM KCl,

Table 2.5 List of Solutions made in the laboratory.

2.2 Methods

2.2.1 Generation of competent prokaryotic cells.

All plasmids used in the present study were amplified in two competent prokaryotic cell types: for most plasmids, Top10 *E.coli* were used and for pCpGL plasmids, DH5α (containing the Pir gene) *E.coli*. To generate a stock of these bacterial cells, the following protocol was used:

A single colony of each type of *E.coli* was picked from an LB agar plate, mixed with 5 ml of LB broth and incubated overnight at 37°C with 225rpm shaking. 1ml of the cell rich LB broth was then transferred into 200ml LB broth and incubated for 2-3 hours at 37°C with 225rpm shaking. To monitor the optical density of the cells, an Eppendorf Biophotometer was used. Once the optical density of the culture reached 0.4, the cells were placed on ice for 10 minutes. After that, the cells were centrifuged at 4k rpm, 4°C for 12min and resuspended in 66.6ml of Buffer I. Another incubation on ice for 15min and refrigerated centrifugation at 4k rpm at 4°C followed. The pelleted cells were then resuspended in 8ml of Buffer II, split into 200µl aliquots, left on ice for 20 minutes, snap frozen using liquid Nitrogen and stored at -80ºC.

2.2.2 Plasmid amplification.

2.2.2.1 Transformation.

A short but efficient method was employed for plasmid transformation into bacterial cells: 100ng of plasmid DNA was mixed with 100µl of competent *E.coli* and incubated on ice for 5min. Preheated LB broth (37ºC) was added to each sample and heat-shocked at 42ºC for 30sec. After that the cells were incubated on ice for 2min. The entire mixture was then plated onto preheated (37ºC) LB agar plates containing 100µg/ml

ampicillin (TOP10) or 25μg/ml zeocin (DH5α) for positive selection. The plates were incubated at 37ºC overnight until colonies had grown.

2.2.2.2 Amplification and extraction.

Cells from a single colony were transferred to 5ml antibiotic containing (100μg/ml ampicillin or 25μg/ml zeocin) LB broth and incubated at 37ºC, 225rpm shaking overnight. Following the incubation, the 5ml of cell rich LB broth was either harvested for low yield plasmid DNA using Qiagen plasmid Mini Prep kit, or was poured into 150ml antibiotic containing LB broth (100μg/ml ampicillin or 25μg/ml zeocin), incubated at 37ºC, 225rpm shaking overnight and then harvested for high yield plasmid DNA using Qiagen plasmid Maxi or Midi Prep kit (according to the manufacturers protocol). The obtained plasmid stocks were quantified for dsDNA concentration with an Eppendorf Biophotometer, analysed using 1% agarose gel electrophoresis and diluted to 100ng/μl.

2.2.3 Polymerase chain reaction.

Polymerase chain reaction was used for generating short DNA segments or for site-directed mutagenesis of the existing plasmids. The following reagents were mixed in 100μl PCR tubes:

10x Pfx enhancer solution	15μl
10x Pfx amplification buffer	5μl
Forward primer (100ng/μl)	1μl
Reverse primer (100ng/μl)	1μl
dNTPs (10mM each)	1μl
Pfx DNA Polymerase	1μl

$MgSO_4$ (50mM)	1µl
Template DNA (100ng/µl)	1µl
ddH_2O	24µl

Each mixture was placed in a Techne TC512 PCR machine with a heated lid at 105°C and set for an initial denaturation of 95°C for 30sec, followed by 25 cycles of 95°C for 30sec, 55°C for 1min and 68°C for 30sec. At the end of the reaction a final extension of 75°C for 7min and a final hold at 4°C was applied.

Following the PCR, either a short DNA segment or a mutated plasmid was obtained. Short DNA segment PCR products were run on a 1% agarose gel and the appropriate band was purified using Promega Gel extraction kit (according to manufacturer's protocol). The purified DNA was then used for subcloning (see chapter 2.2.4). Whereas, the plasmid DNA products were amplified at a low yield (see chapter 2.2.2), to first check for errors by sequencing at Eurofins MWG Operon. Error-free plasmids were then amplified at a high yield (see chapter 2.2.2) and used as stock.

2.2.4 Subcloning.

Short DNA PCR products or ordered DNA sequences (in parent plasmids), as well as the destination plasmids (either pcDNA3 or pGL3 enhancer) were digested with appropriate enzymes at 37°C for 1 hour, run on a 1% agarose gel and purified using Promega Gel extraction kit (according to manufacturers protocol). KpnI and HindIII restriction enzymes were used for subcloning into pGL3 enhancer, while, BamHI and EcoRI were used for subcloning into pcDNA3 vector. RE buffer 4 was used for all reac-

tions. Ligation reactions were incubated in an ice-bath placed at a room temperature overnight (for a wide range of temperature). 0 (no insert), 1:1, 1:3 or 1:6 molar ratios of vector:insert were incubated overnight with 100ng of destination plasmid, 1.5µl of T4 DNA ligase and 4µl of 5x T4 DNA ligase buffer, in a final reaction volume of 20µl. The purified insert's amount was determined by the formula:

Insert mass = molar ratio of the insert x vector mass x insert length / vector length

Where insert and vector mass is in nanograms, length is in base pairs and the molar ration is either 0, 1, 3 or 6. Following the overnight incubation, the ligation mixtures were transformed into the appropriate *E.coli* strain (see chapter 2.2.2.1). Several colonies were picked from a plate that had most colonies compared to no insert (negative control) plate. Plasmid DNA was extracted (at a low yield) from selected colonies as described previously in chapter 2.2.2 and sent for sequencing to Eurofins MWG Operon. Error-free plasmids were then amplified at a high yield (see chapter 2.2.2.2).

2.2.5 Cell culture.

All cells were grown in a humidified incubator with a 5% CO_2 supply at 37ºC. HeLa cells, HEK 293 and HEK 293T cells were cultured in DMEM media with 10% FCS, L-Glutamine and AA. HEK 293T EBV ZKO cells (Delecluse et al., 1998) were grown in RPMI media with 10% FCS, Hygromycin B and L-Glutamine. Raji and AK31 cells were maintained in RPMI media with 10% FCS and PSG. For long term storage of cell lines, exponentially growing cells were washed and resuspended in 90% Foetal Calf Serum supplemented with 10% DMSO at $1x10^7$cells/ml. Cells were frozen at -80°C for

several days before long term storage in liquid nitrogen. Frozen cell stocks were recovered by rapid thawing, followed by a subsequent wash in 10ml of chilled media before culturing.

2.2.6 Transfections.

HeLa, HEK 293, HEK 293T or HEK 293T ZKO adherent cells were transfected using the Effectene transfection reagent kit (Qiagen) in a class II microbiology cabinet. Cells were washed, re-suspended in PBS, counted and $4x10^5$ cells were added to 3.5cm diameter plates (of a 6-well plate) and incubated for 24 hours in 3ml of the appropriate media. Following the incubation, the existing media was replaced by 2.2ml of fresh media containing a total of 1µg of vector DNA (of 100ng/µl stock) mixed (by vortexing) with Effectene reagents (2.5µl Effectene, 8µl Enhancer and 100µl Buffer EC). N.B. HEK 293T ZKO were grown in medium flasks (75cm²) and the cell numbers, DNA and reagents volumes were appropriately scaled up to 8x of the amounts described above. Depending on the experiment, the cells were incubated for 48 or 72 hours before harvesting.

Raji and AK31 suspension cells, were transfected by electroporation. The cells were counted, spun and resuspended in appropriate media at the concentration of $4x10^7$ cells/ml. 0.25ml ($1x10^7$ cells) were added to each VWR electroporation cuvette together with 10µg of desired plasmids (of 100ng/µl stock), and pulsed at 250V at a capacitance of 975Ω on a Bio-Rad Gene Pulser II electroporator. The contents of the cuvettes were then transferred to a small flask (25cm²) containing 5ml of RPMI and incubated for 48 hours before harvesting.

2.2.7 SDS-PAGE.

Transfected cells were harvested, washed in DPBS, and resuspended in 200µl 8M Urea. The resuspended cell pellet was then sonicated 3x10sec at 25% amplitude on a Sonics, Vibra Cell sonicator with Branson 250 Microtip. 10µl of each obtained sample was mixed with 2xPSB, boiled for 5min and run on a NuPAGE Bis-Tris Precast Gel with MOPS buffer. Electrophoresis was carried out for 50min at 200V.

2.2.8 Western blotting.

Following the SDS-PAGE, proteins samples were transferred onto a Protran nitro-cellulose membrane, which was then blocked with 5% milk or with Odyssey blocking buffer. Primary antibody was left to bind at 4°C overnight followed by three washes with PBS + 0.1% Tween 20. Two different types of secondary antibodies were used but both were left to bind for 1 hour, followed by three washes with PBS + 0.1% Tween 20. If a IRDye infrared fluorescent secondary antibody was applied, the membranes were directly developed on a Li-C or Odyssey Fc Dual-Mode Imaging System. If a HRP-linked secondary antibody was used, the membranes were washed in 1ml ECL I solution + 1ml ECL II solution, exposed in the dark room to a photographic film and developed in a X-ograph Compact X2 developer.

2.2.9 Benzonase lysis.

HEK 293T cells from small 6-well plates were harvested (with a DPBS wash) and pelleted, 72h post-transfection. The samples were then resuspended in 100µl of Benzonase buffer with 1µl of Benzonase nuclease and incubated on ice for 30min. 2µl of 5M NaCl was then added to each tube and left on ice for 15min. Finally the samples were spun for 10min at 13,000rpm, 4ºC and the supernatant retained. 10µl of supernatant was

mixed with 2xPSB and analysed by western blotting (see chapter 2.2.7), while the remainder of the sample was used for Flag pulldowns.

2.2.10 Flag pulldowns.

20µl of anti-Flag gel (for each sample) was washed with 500µl of Benzonase buffer, and resuspended in 40µl Benzonase buffer. The benzonase lysis samples (see 2.2.8) were then added to the anti-Flag gel and left rotating overnight at room temperature (RT). The next day, the samples were centrifuged for 30sec at 13,000rpm and the supernatant discarded. Following 2x Benzonase buffer washes, the anti-Flag gel was resuspended in 20µl of 2xPSB, boiled for 5min and analysed by western blotting (see chapter 2.2.7).

2.2.11 Luciferase assays.

All cell lines cultured for the luciferase assay were incubated for 48 hours post-transfection. Upon harvesting, the cell suspension was centrifuged at 2k for 5min (adherent cells were scraped first). The cell pellet was resuspended and split into 2x500µl DPBS. Half of the samples were used for western blotting (see chapter 2.2.7). The other half was dissolved in 250µl of Passive Lysis Buffer (provided by Promega) and incubated at room temperature for 15 minutes. The lysed cells were then centrifuged for 10min at 8k rpm and the supernatant was pipetted into clean tubes and stored at -20ºC if needed. For the luciferase activity analysis, 10µl aliquots of each lysate sample were pipetted into a 96-well white luminescence plate and analysed using 50µl Promega luciferase detection kit reagents (luciferase substrate + luciferase buffer) on a Promega Glomax multidetection system. For normalisation purposes, 200µl of 1xBio-Rad protein

assay was added to 10µl of lysates in a 96-well transparent plate and analysed on the same machine.

2.2.12 Chromatin Immunoprecipitation.

2.2.12.1 Cross-linking and chromatin preparation.

Chromatin immunoprecipitations were carried out from HEK 293T EBV ZKO cells which were incubated in medium flasks (75cm²) 48 hours post-transfection. Upon completion of incubation time, the growth media was carefully replaced with new media containing 1% final concentration of formaldehyde (for cross-linking) and left for 15min at room temperature. 0.125M final concentration of glycine was then added to the cells, which were then scraped, spun and washed with DPBS. (From here on the samples were handled on ice). Each sample of pelleted cells were then resuspended in 600µl Cell Lysis Buffer and left on ice for 10min. The lysates were then centrifuged at 8k rpm for 5min at 4ºC and the supernatant removed. Obtained nuclei (pellet) were then mixed with 200µl SDS lysis buffer and sonicated 10x10sec at 30% amplitude on a Sonics, Vibra Cell sonicator with Branson 250 Microtip. Finally, the samples were quantified for total protein concentration (Warburg-Christian method) with the Eppendorf Biophotometer, snap frozen in liquid Nitrogen and stored at -80ºC.

2.2.12.2 Beads preparation.

Both protein A-sepharose and protein G-sepharose beads were used for ChIP. 28µl protein A-sepharose slurry + 28µl protein G-sepharose slurry + 54µl ChIP dilution buffer were mixed (per sample of nuclear extract obtained earlier). The beads were then washed twice in ChIP dilution buffer prior to the addition of 55µl ChIP dilution buffer

supplemented with 55µl of salmon sperm DNA and rotated for 30min at 4°C. Finally the beads were washed twice and resuspended in ChIP dilution buffer in a total volume of 100µl per sample.

2.2.11.3 Chromatin precipitation.

50-100µl (proportionate to protein quantification) of each sample prepared in chaper 2.2.12.1 was diluted with ChIP dilution buffer to a final volume 1ml, of this 40µl was retained and stored at -20°C as Input DNA. 45µl of prepared beads slurry (from chapter 2.2.12.2) was then pipetted to each 960µl sample and left rotating for 30min at 4°C. The samples were centrifuged and the supernatant was pipetted into a tube with 2µg of ScZ antibody, which was then left rotating for 60min at 4°C. Following incubation, a new aliquot of 55µl of prepared bead slurry (from chapter 2.2.12.2) was mixed with the samples which were again left rotating overnight at 4°C. The following day a series of 4 washes of 5min rotating at 4°C followed (in sequence; volume per sample): 1ml low salt buffer; 1ml high salt buffer; 1ml LiCl buffer and 1ml TE buffer. After the washes, the beads were either directly resuspended in 100µl 2xPSB and analysed using SDS-PAGE and western blotting (see chapter 2.2.7 and 2.2.8) or eluted with 100µl elution buffer. At this point the Input DNA samples were also mixed with 60µl elution buffer, and together with eluted samples, were left to cross-link overnight at 65ºC. In the morning all the samples (including Input DNA and ChIP samples) were mixed with 150µl TE buffer, 5µl Proteinase K and incubated for 2h at 55ºC. Finally the samples were stored at -20ºC.

2.2.11.4 Relative quantification analysis.

The ChIP samples were analysed on a 7500 Real Time PCR System from Applied Biosystems. A standard curve consisting of a serial dilution of Input DNA of one sample was run along all the ChIP samples and all Input DNA samples (1/2 diluted), with oriLyt flank (7/8) or Rp3 primers. Each well of the PCR plate was filled with the following contents:

GoTaq qPCR master mix	12.5µl
Forward primer	2.5µl
Reverse primer	2.5µl
Sample (Input or ChIP)	2.5µl
ddH_2O	5µl

Cycling conditions were applied to a qPCR plate: 95°C for 30min, followed by 40 cycles of 95°C for 30sec, 60°C for 1min and 72°C for 30sec, with a final denaturation stage of 95°C for 1min. The obtained data for each ChIP sample was then divided by its corresponding Input DNA value for normalisation.

2.2.12 *In vitro* transcription.

In vitro transcription was achieved with the help of RiboMax T7 Promega kit according to manufacturer's protocol. The obtained RNA was quantified with the Eppendorf Biophotometer and diluted to 100ng/µl.

2.2.13 *In vitro* translation.

In vitro translation was performed using Rabbit Reticulocyte Lysate system (Promega). The following components were mixed for each sample:

Rabbit Reticulocytes	35µl
Amino acid mix 1mM (minus Methionine)	1µl
[^{35}S] Methionine	2µl
RNasin	1µl
RNA substrate	1µl
ddH_2O	10µl

The mixture was then incubated at 30°C for 90min. Following the incubation, 10µl of each sample were run on a SDS-PAGE gel, transferred onto a nitrocellulose membrane and exposed to a Molecular Dynamics Storage Phosphor screen for 24h. The samples were then visualised on a Storm 860 phosphorimager and each protein band was quantified. The remaining samples were put through a DNA affinity chromatography.

2.2.14 DNA affinity chromatography.

2.2.14.1 Beads and oligonucleotides preparation.

For each sample, 10µl of M-270 Streptavidin Dynabeads were aliquoted, washed three times in the provided 2x B&W buffer (10mM TrisHCl pH7.5, 1mM EDTA, 2M NaCl) and resuspended in 10µl of 2x B&W buffer. In the meantime, 200µM of forward Rp-biotin or Rp mut-biotin oligos were incubated together with 400µM reverse com-

plement primers (unbiotinylated) at 95°C for 2min, 65°C for 10min and 37°C for 30min. Following the incubation, 5µl of 10x diluted double-stranded primers (20µM) were pipetted to each beads sample and left to bind at RT for 15min. Three washes with 1x B&W buffer followed, and the beads (bound with DNA) were resuspended in 7µl binding buffer.

2.2.14.2 Pulldowns.

In vitro translated proteins from chapter 2.2.13 were added to the beads-biotin-DNA prepared in chapter 2.2.14.1. The volume of each *in vitro* sample added was inversely proportionate to the band intensity quantified in chapter 2.2.13: 2µl of his-Zta; 6.26µl of his-Zta K12R and 2.4µl of his-SUMO2-Zta. The proteins were left to bind the beads-biotin-DNA for 30min at RT, after which the beads were washed three times with 100µl D150. Finally, the beads were resuspended in 20µl 2xPSB, boiled at 95°C for min and run on a SDS-PAGE. The samples were then transferred to a nitrocellulose membrane and exposed to a Molecular Dynamics Storage Phosphor screen for at least 24h. The samples were visualised on a Storm 860 phosphorimager and each protein band was quantified.

3. Molecular interplay between Zta, VPK and the DNA damage response pathway.

3.1 Introduction.

The necessity to reactivate lytic cycle and produce new virion particles is a trade-off for EBV. With the switch from latency comes the elevated risk of detection by cellular molecular machinery, which in turn can create unfavourable conditions for viral replication. One such host mechanism is the activation of the DNA damage response (DDR) during EBV's lytic phase (Kudoh et al., 2005). The DDR pathway is able to activate cell cycle checkpoints or induce apoptosis, events that would be detrimental to efficient EBV replication. One possible reason for activation of the DDR, is that the amplified EBV genome could be mistaken as damaged host DNA. This theory is supported by co-localisation of DDR proteins Mre11 and Nbs1 with EBV replication compartments (Kudoh et al., 2005). On the other hand, a number of lytic proteins have been identified that can trigger the activation of the DDR pathway in the absence of viral genome replication. The immediate-early protein Rta is sufficient to induce typical hallmarks of the DDR such as histone H2AX phosphorylation and p53 up-regulation in epithelial cells (Chen et al., 2009). EBV's DNase encoded by *BGLF5* was shown to cause double-strand breaks and increase genomic instability, thus elevating the levels of phosphorylated H2AX (γH2AX) in epithelial cells (Wu et al., 2010).

Recently, however, it has emerged that there might be an advantage to the activation of the DDR during EBV lytic cycle. The viral protein kinase (VPK) and its homologue orf36 of murine γ-herpesvirus 68 (γHV68) are able to induce γH2AX levels inde-

pendently of other viral proteins. Orf36, ATM and H2AX phosphorylation are all required for genome replication of γHV68 in mice (Tarakanova et al., 2007). VPK was also found to be required for efficient production of infectious EBV virions and expression of some lytic viral genes (Feederle et al., 2009; Gershburg et al., 2007). Li et al. (2011) showed that both of these processes are at least in part reliant on VPK directly phosphorylating the histone acetyltransferase TIP60. Phosphorylated TIP60 can then acetylate histones or activate the ATM protein, which in turn phosphorylates H2AX. Histones with the euchromatic acetylation marks and γH2AX at the EBV lytic promoters and at the oriLyt could aid lytic gene transcription and viral DNA synthesis (Li et al., 2011).

EBV can actively recruit parts of the DDR pathway, however, the downstream effects of DDR might not be favourable for the virus. The programmed DNA repair stimulated by the ATM pathway could adversely modify the viral genome, or alternatively, could trigger p53 mediated apoptosis; both outcomes detrimental to EBV lytic replication. A likely candidate to alleviate these unwanted effects of DDR is Zta. The ability of Zta to bind and label p53 for proteasomal degradation could counteract the potential negative impact of ATM on viral replication (Sato et al., 2009a; Sato et al., 2009b). Furthermore, the ability of Zta to activate the lytic cycle was shown to be dependent on its ability to bind 53BP1, a scaffold protein, heavily involved in the ATM pathway, suggesting a protective role for this binding (Bailey et al., 2009; Lee et al., 2010). Thus, it is plausible to assume that VPK recruits DDR machinery needed for efficient EBV replication and viral transcription, whereas Zta inhibits some of its downstream effectors to prevent the damaging effects of DDR.

Interestingly, VPK and Zta were also reported to interact with each other forming a stable complex. This complex requires VPK phosphorylation of Zta on Serine 209. Moreover, VPK seems to down-regulate the transactivation activity of Zta. An increase of VPK protein levels during the lytic cycle also correlates with a decrease in Zta's mRNA levels (Asai et al., 2006; 2009). Finally, it was also shown that Zta can be post-translationally modified by SUMOylation on Lysine 12, adding another possible level for the fine tuning of Zta's function (Adamson and Kenney, 2001).

The aim of this chapter was to investigate the following question:

- How do VPK and Zta affect H2AX phosphorylation in human epithelial cells?
- Is Zta targeted for proteasomal degradation?
- Does VPK affect Zta's SUMOylation?
- Is VPK phosphorylation and reported binding important for Zta's SUMOylation?

3.2 Results.

3.2.1 VPK and Zta have no effect on H2AX phosphorylation in HEK 293T.

VPK induced H2AX phosphorylation has been shown to occur in murine fibroblasts (Tarakanova et al., 2007) and a human EBV positive Burkitt's lymphoma cell line, Akata (Li et al., 2011). However, it is not known whether this also occurs in human epithelial cells. To investigate this, HEK 293T cells were transfected with Flag-VPK, the same Flag-tag expression vector used by Tarakanova et al. (2007). This was done to allow immunoblot detection by anti-Flag antibody, as there were no available VPK antibodies on the market at the time of this study. The presence of VPK and phosphorylated H2AX were detected after 24, 48 or 72 hours post-transfection. The immunoblot data was then quantified and normalised to an actin control, as represented in panel B of Figure 3.1. The data [Figure 3.1] show that the levels of β actin remained relatively constant compared to the levels of Flag-VPK and γH2AX which rose steadily as the experiment progressed throughout the timecourse. However, no significant increase in γH2AX levels in response to VPK could be observed at any of the time points [Figure 3.1].

To test if the phosphorylation of H2AX was dependent on Flag-VPK plasmid levels, a series of titrations of Flag-VPK from 0.05μg to 1μg was transfected into HEK 293T. The abundance of the proteins was determined 72 later and the immunoblot data [Figure 3.2 (A)] was quantified and normalised in the same manner as before. Once again, no significant change of γH2AX at any VPK level transfected was observed [Figure 3.2].

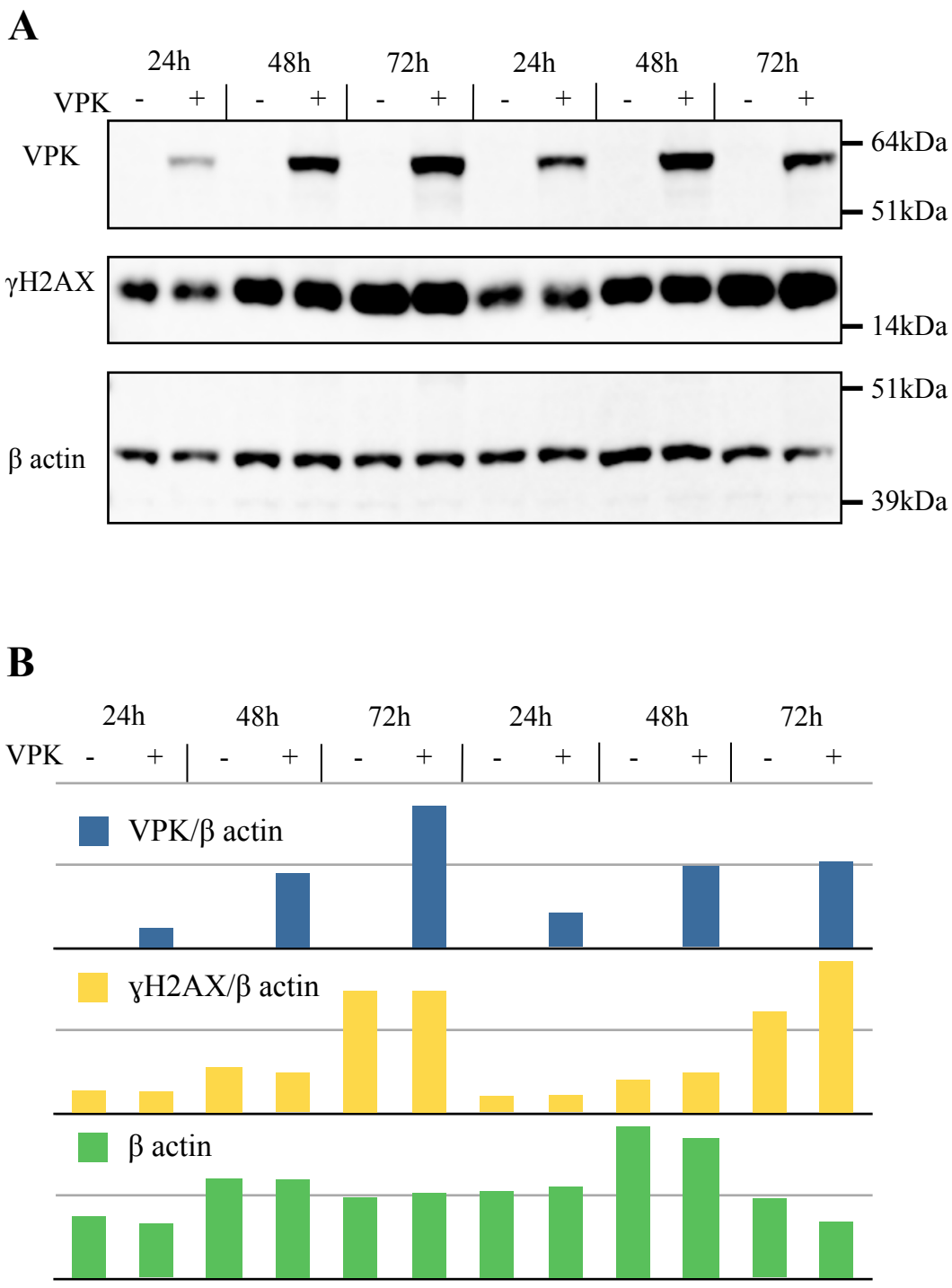


Figure 3.1 Time-course of VPK effects on H2AX phosphorylation.
HEK 293T cells were transfected with 1µg Flag-VPK (+) or with an empty vector (-) and harvested at 24, 48 or 72 hours (with repeats) post-transfection. The harvested cells were then resuspended in 8M Urea and sonicated. **A.** Western blotting results using anti-phospho H2AX (γH2AX), anti-Flag (VPK) and anti-β actin antibodies. **B.** The quantified relative intensity of the bands for each antibody. VPK and γH2AX were normalised to the corresponding β actin values.

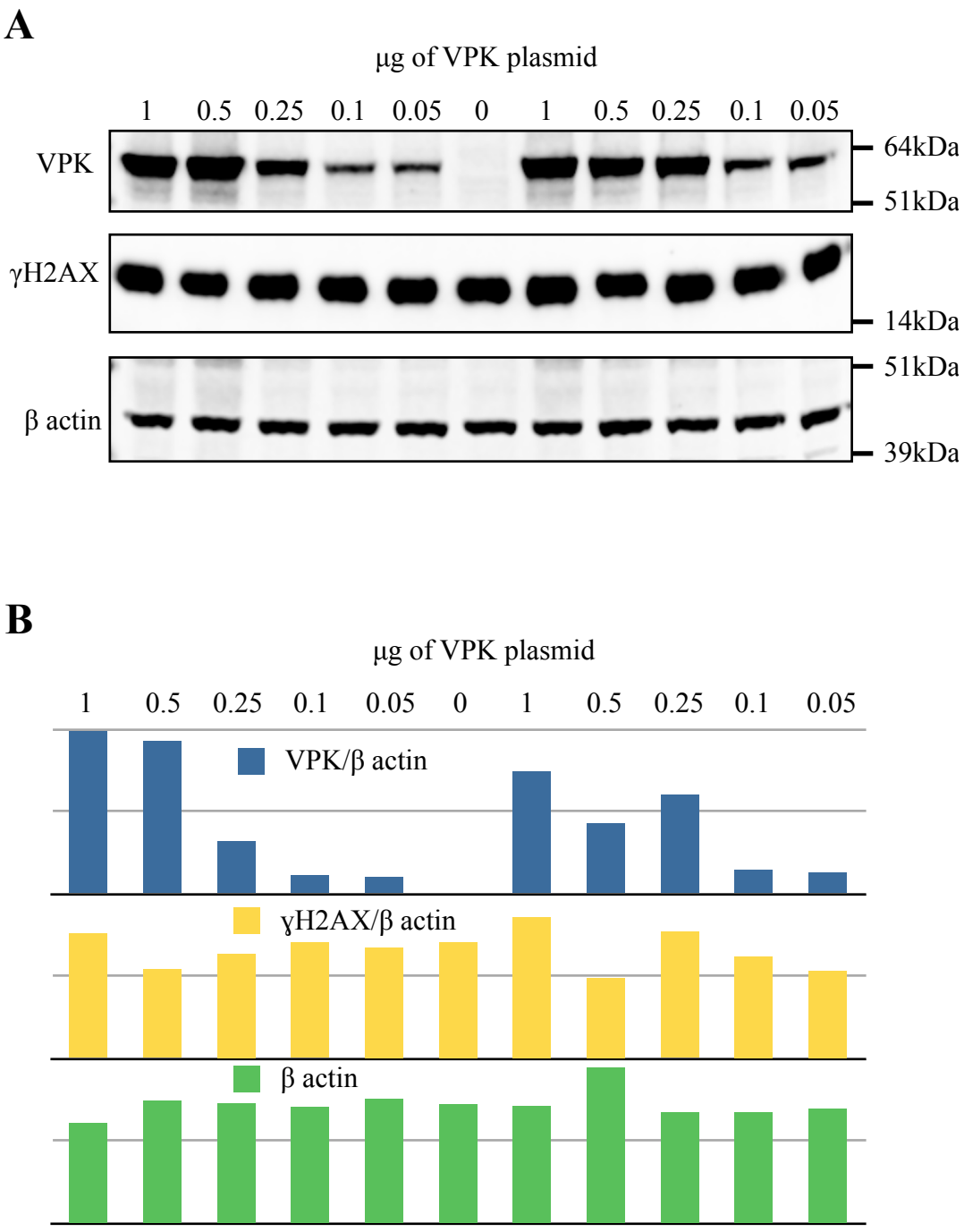
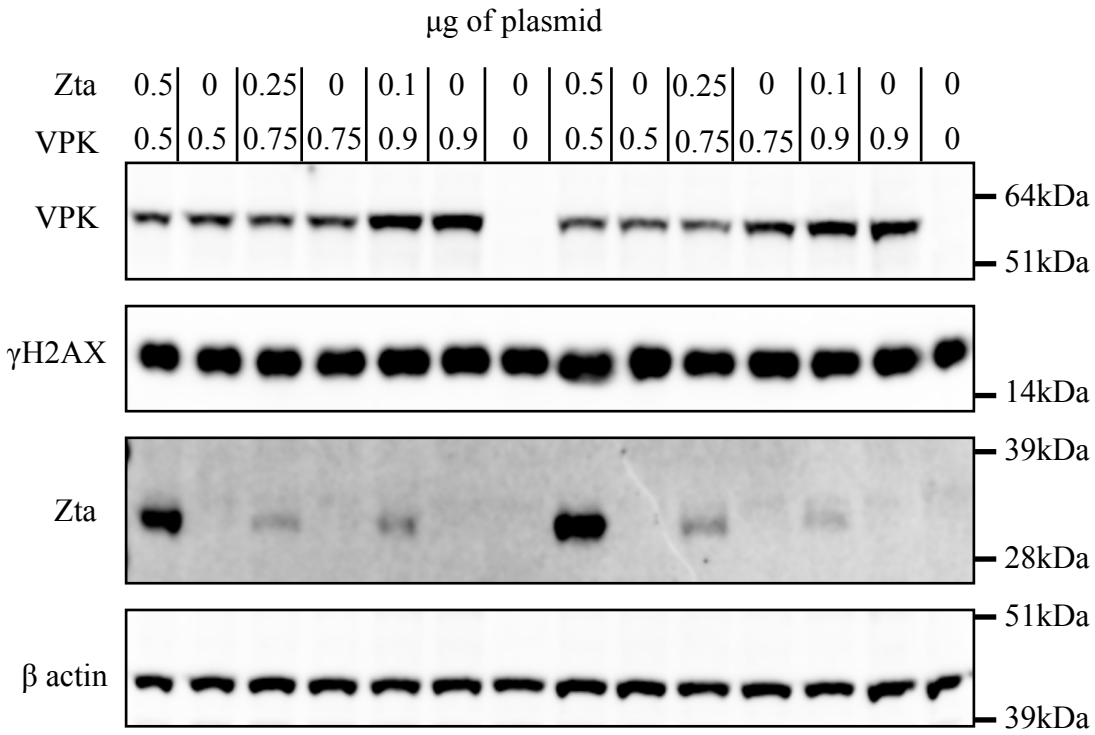


Figure 3.2 Effects of VPK titration on H2AX phosphorylation.
HEK 293T cells were transfected with various amounts Flag-VPK and harvested at 72 hours (with repeats) post-transfection. The harvested cells were then resuspended in 8M Urea and sonicated. **A.** Western blotting results using anti-phospho H2AX (γH2AX), anti-Flag (VPK) and anti-β actin antibodies. **B.** Quantified relative intensity of the bands for each antibody. VPK and γH2AX are normalised to the corresponding β actin values.

A


μg of plasmid
Zta 0.5 0 0.25 0 0.1 0 0 0.5 0 0.25 0 0.1 0 0
VPK 0.5 0.5 0.75 0.75 0.9 0.9 0 0.5 0.5 0.75 0.75 0.9 0.9 0
VPK
64kDa
51kDa
γH2AX
14kDa
Zta
39kDa
28kDa
β actin
51kDa
39kDa

B

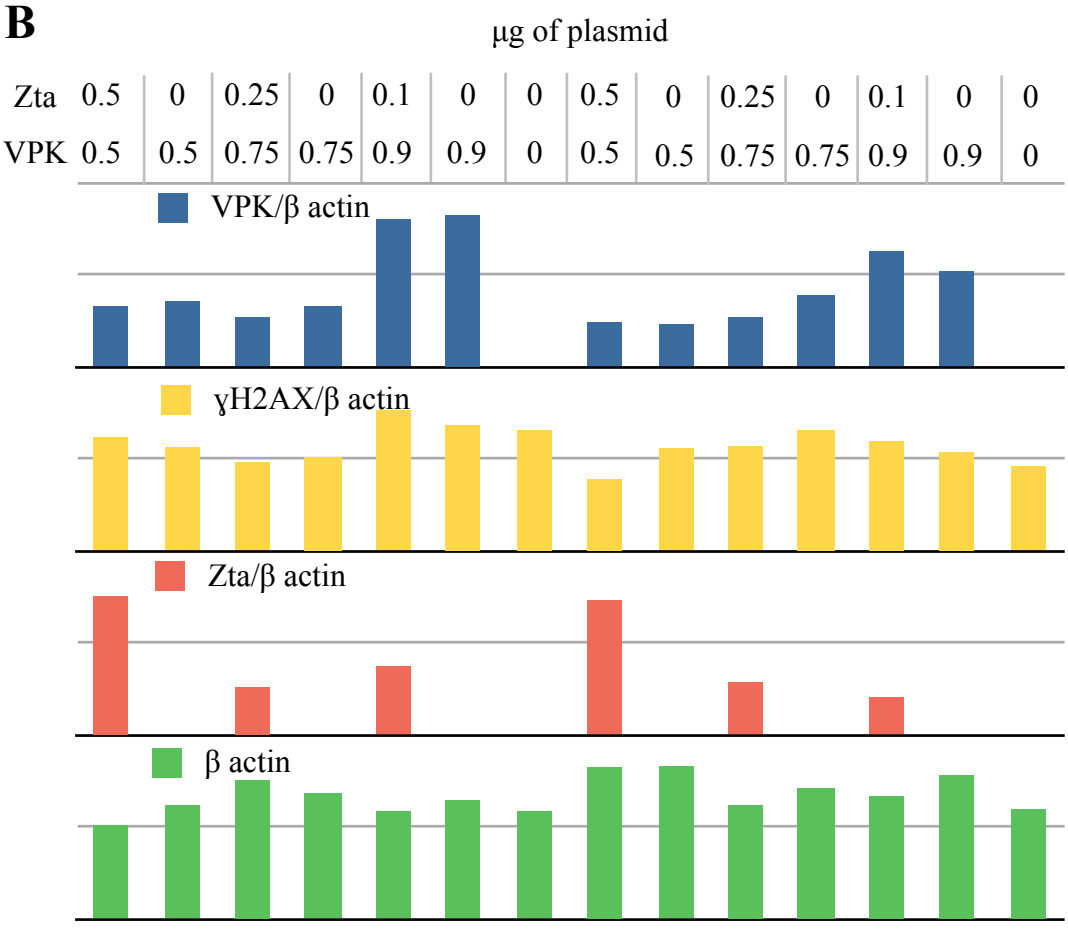

μg of plasmid
Zta 0.5 0 0.25 0 0.1 0 0 0.5 0 0.25 0 0.1 0 0
VPK 0.5 0.5 0.75 0.75 0.9 0.9 0 0.5 0.5 0.75 0.75 0.9 0.9 0
VPK/β actin
γH2AX/β actin
Zta/β actin
β actin

Figure 3.3 Effects of VPK and Zta titrations on H2AX phosphorylation.
HEK 293T cells were transfected with various amounts Flag-VPK and his-Zta and harvested at 72 hours (with repeats) post-transfection. The harvested cells were then resuspended in 8M Urea and sonicated. **A.** Western blotting using anti-phospho H2AX (γH2AX), anti-Flag (VPK), BZ1 (Zta) and anti-β actin antibodies were used. **B.** Quantified relative intensity of the bands for each antibody. VPK, γH2AX and Zta are normalised to the corresponding β actin values.

These results to some extent contrast with Tarakanova et al. (2007) and Li et al. (2011) publications, which saw an increase in the levels of phosphorylated H2AX in response to transfected VPK. However, Tarakanova et al. (2007) studies were performed in a murine cell line model and Li et al. (2011) used human lymphocytes, both of which differ from the cellular background used in the present study.

The Li et al. (2011) results suggest a possible involvement of other EBV proteins in the induction of H2AX phosphorylation, as the observed increase in γH2AX occurred in an EBV infection background (Li et al., 2011). Since Zta is a major modulator of both viral and host transcription, and was shown to directly interact with VPK, it was decided to co-transfect the two proteins together to study the effects on H2AX phosphorylation in HEK 293T cells. As shown in Figure 3.3, γH2AX levels did not change significantly in response to any amount of his-Zta or Flag-VPK transfected, indicating the lack of involvement of either or both proteins in manipulation of this part of the DDR pathway in HEK 293T cells [Figure 3.3].

3.2.2 Zta is not targeted for proteasomal degradation.

Zta was reported to be post-translationally modified by Adamson and Kenney (2005). To identify these modifications, Zta and his- tagged Zta were transfected into

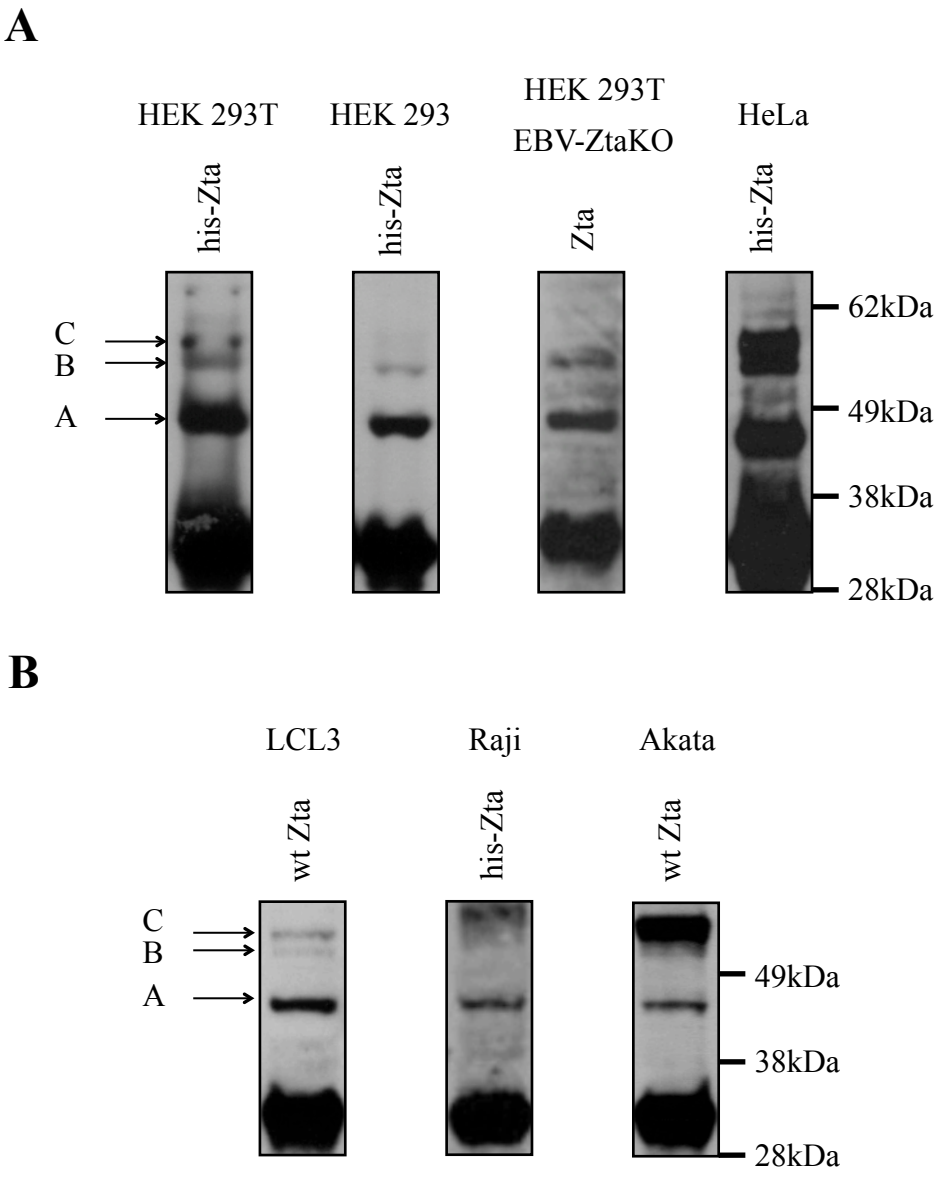


Figure 3.4 Comparison of different Zta PTMs in various cell lines.
A. Zta's PTMs in epithelial cells. HEK 293, 293T, 293T EBV-ZtaKO and HeLa cells were transfected with 1µg of his-Zta (in pcDNA3 vector) or Zta (in pBabe vector) and harvested 72h post-transfection. **B.** Zta's PTMs in B-cells. Akata cells were incubated for 48h post induction of lytic cycle with IgG. Raji cells were incubated for 48h post-transfection with his-Zta (in pcDNA3 vector). LCL3 cells are spontaneously lytic. All the samples were analysed by western blotting using BZ1 antibody. Note the three main higher molecular weight forms of Zta marked by the arrows and denoted as A, B and C. LCL3 and Akata samples were kindly provided by my lab colleague Sharada Ramasubramanyan.

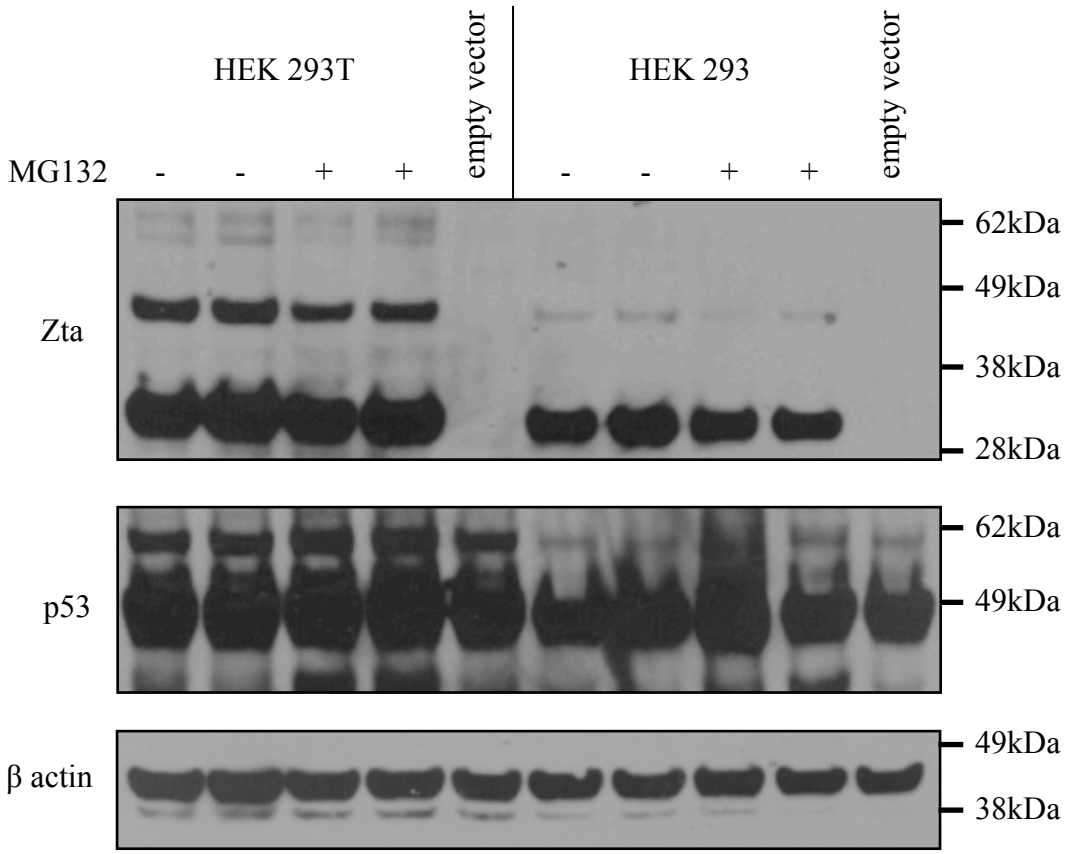


Figure 3.5 Effects of proteasomal inhibition on Zta in HEK 293 and HEK 293T cells.
HEK 293T or HEK 293 cells were transfected with his-Zta (in pcDNA3), after 72h half of the samples were incubated with MG132 (10μg/ml final concentration in the media) for 8 hours. The cells were then harvested in 8M Urea, sonicated and analysed by western blotting using anti-β actin, DO1 (p53) and BZ1 (Zta) antibodies.

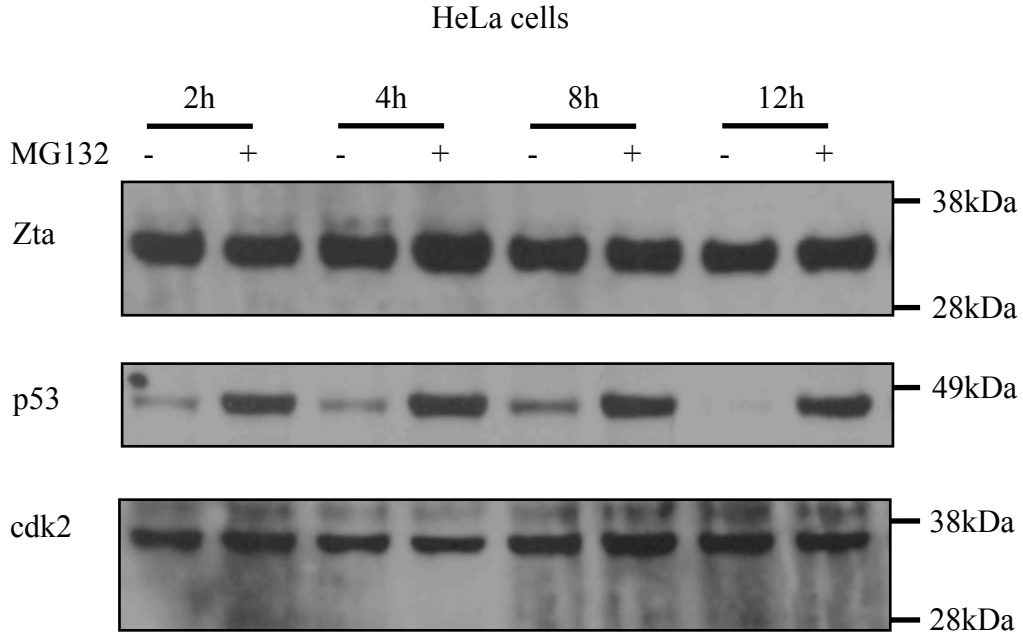


Figure 3.6 Effects of proteasomal inhibition on Zta in HeLa cells.
HeLa cells were transfected with Zta (in pBabe), after 72h half of the samples were incubated with MG132 (10µg/ml final concentration in the media) for 2, 4, 8 or 12 hours. The cells were then harvested in 8M Urea, sonicated and analysed by western blotting using anti-cdk2, DO1 (p53) and BZ1 (Zta) antibodies.

epithelial and B cells, or the wild type Zta from EBV infected B-cells was observed. HeLa cells are a cervical carcinoma derived cell line, whereas HEK293 and HEK293T originate from human embryonic kidney cells. These cell lines are a commonly used model to study EBV replication. HEK 293T EBV-ZKO, on the other hand, are infected with a functional EBV genome; however these cells are not able to undergo lytic cycle, as the *BZLF1* gene is disrupted by insertional mutagenesis. These cells provide a model of EBV infection in epithelial cells and are lytic cycle inducible upon transient transfection with Zta. LCL3 is a lymphoblastoid cell line that is spontaneously lytic and therefore produces Zta at a constant rate. Akata cells can be induced with such agents as IgG immunoglobulins to initiate the lytic cycle and Zta expression, providing a convenient switch for investigating EBV lifecycle. Raji cells are also a Burkitt's lymphoma cell line that contain an EBV genome with various deletions and can be activated to undergo early stages of the lytic cycle by Zta transfection. As can be seen from Figure 3.4, Zta has at least 3 different higher molecular weight forms denoted as A, B and C. The fact that the PTMs can be observed in both epithelial and B cell lines, irrespective of EBV presence, suggests that the post-translational modifications related to higher molecular forms of Zta are not dependent on viral proteins, and are not cell type specific. In addition to that, the N-terminal 6xhistidine tag had no effect on the levels of the higher molecular forms of Zta [Figure 3.4]. However, it is important to note the different relative levels of abundance of the A, B and C forms in various cell lines. A higher subset of the Zta protein pool seemed to be post-translationally modified HEK 293, HEK 293T and HeLa compared to LCL3 and Raji [Figure 3.4]. Nevertheless, Akata cells exhibited considerable amounts of the C form of Zta PTM, which corresponds to a multimeric modification [Figure 3.4].

It is possible that at least some of the higher molecular forms of Zta could be caused by Ubiquitylation. Because one of the main functions of Ubiquitin is to target proteins for proteasomal degradation, it was decided to test if Zta's turnover is affected by this mechanism. The HEK 293T and HEK 293 cells were transiently transfected with his-Zta and 72 hours later the cells were incubated with a proteasomal inhibitor MG132 for 8 hours [Figure 3.5]. By inhibiting the proteasome, MG132 can elevate the levels of a protein whose turnover is controlled by proteasomal degradation. p53 is one such protein and was used as a positive control in our experiments [Figure 3.5, Figure 3.6]. Data in Figure 3.5 show that Zta levels were not affected in response to the addition of MG132 in HEK 293T or HEK 293 cells. The levels of p53 were also unchanged, suggesting that the MG132 treatment was unsuccessful. [Figure 3.5]. The effect of proteasomal inhibition on Zta was also examined in another cell line, HeLa [Figure 3.6]. Here data show a clear build up of p53 levels in response to incubation with MG132. However, the protein levels of Zta were unchanged once again. Together, these findings strongly suggest that proteasomal degradation is not involved in Zta's turnover.

3.2.3 Zta's PTMs are not dependent on the extreme carboxy terminus of the protein.

As Zta's extreme carboxy terminus (CT) has been shown to be important for viral replication and interaction with the DDR (Bailey et al., 2009), it was decided to investigate the whether CT region influenced the post-translational modifications of Zta. Two mutant versions, Zta AAA and Zta 243ter [Figure 3.7 (A)] were introduced into HEK 293 EBV-ZKO cells and 72 hours later the protein and PTM levels were compared. This revealed a similar pattern of Zta AAA and Zta 243ter bands compared to unmodified

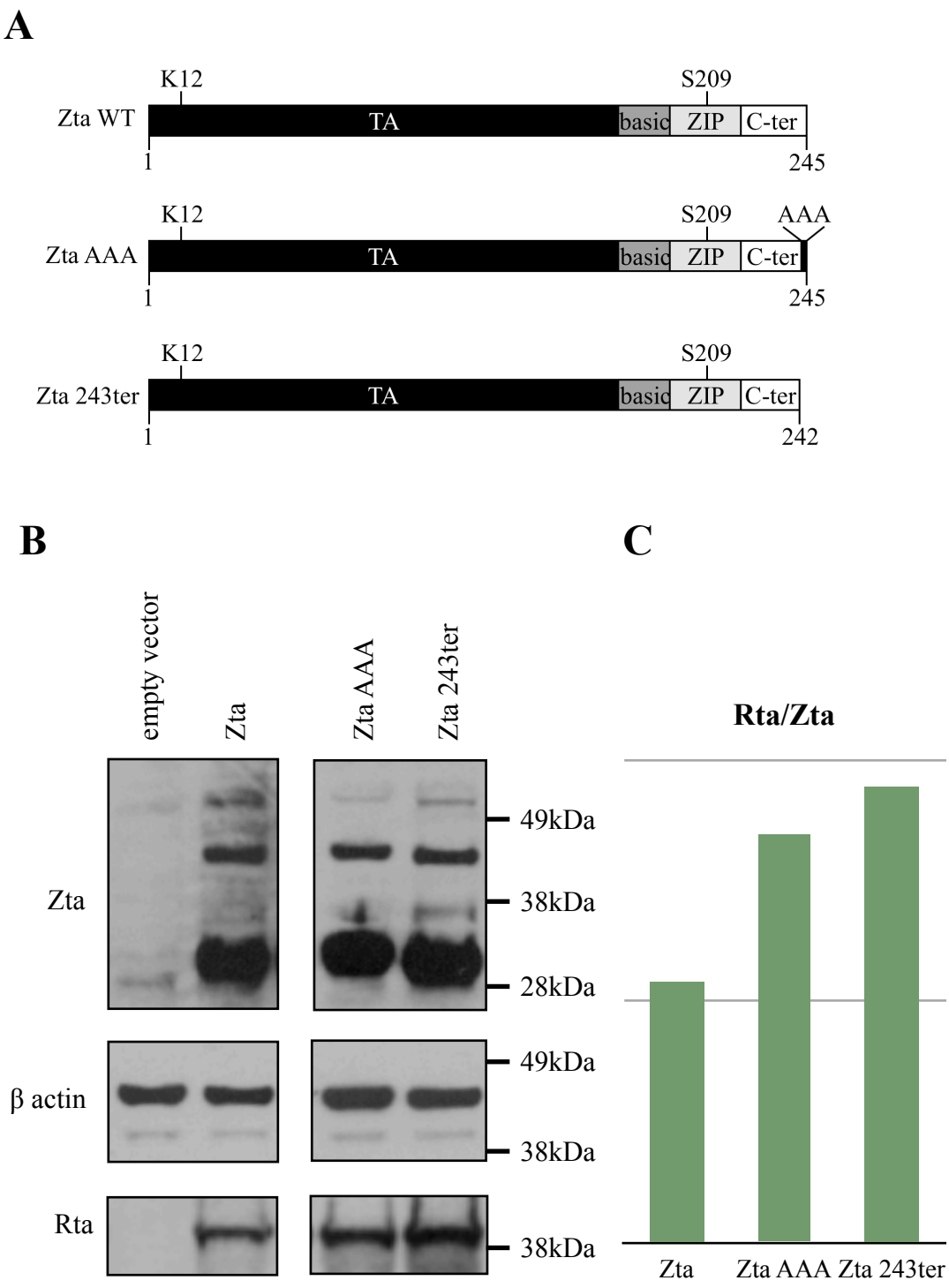


Figure 3.7 Role of C-terminus in post-translational modification of Zta. **A.** Graphical representation of the Zta extreme caboxy terminal mutant constructs used. **B.** HEK 293T EBV Z-KO cells were transfected with 1µg empty pBabe vector, wild type Zta, Zta AAA mutant (last 3 residues mutated to alanine) or the Zta 243 ter mutant (all in a pBabe plasmid). The cells were then harvested 72 hours post-transfection and analysed by western blotting using anti β actin (loading control), anti-Rta and BZ1 (Zta) antibodies. **C.** Quantified Rta levels normalised to Zta protein levels.

Zta [Figure 3.7 (B)], suggesting that the CT region had no significant effect on the Zta's post-translational modifications. In addition to that, the protein levels of Rta were monitored in all transfections. The data show [Figure 3.7 (B)] that Rta expression levels increased when the Zta 243ter and Zta AAA mutants were transfected, indicating that the C-terminus might play a role in Zta's ability to initiate the early lytic transcription of EBV. Together with Bailey et al. (2009), in which it was shown that the same mutant constructs exhibited drastic drops in EBV replication, the data suggest that the extreme carboxy terminus affects both Zta's ability to initiate replication and its effectiveness as a transcription factor.

3.2.4 Lysine 12 is the site of Zta's PTMs.

Adamson and Kenney (2001) showed that mutating Lysine 12 together with Phenylalanine13 of Zta abolished the post-translational modifications of Zta. Lysine residues are known to be the typical sites of covalent attachments of small proteins such as SUMO and Ubiquitin. A K12R point mutant of Zta was generated [Figure 3.8]. Mutating Lysine12 alone was sufficient to abrogate the expression of all higher molecular forms of Zta [Figure 3.9]. Additionally, Rta protein levels increased when Zta K12R was expressed, indicating that Lysine 12 and potentially Zta's PTMs have an inhibitory effect on Zta's transactivation.

A

BZLF1 DNA SEQUENCE ATG…TAA (738 NT)

ATGATGGACCCAAACTCGACTTCTGAAGATGTAAAATTTACACCTGACCCATACCAGGT
GCCTTTTGTACAAGCTTTTGACCAAGCTACCAGAGTCTATCAGGACCTGGGAGGGCCAT
CGCAAGCTCCTTTGCCTTGTGTGCTGTGGCCGGTGCTGCCAGAGCCTCTGCCACAAGGC
CAGCTAACTGCCTATCATGTTTCAACCGCTCCGACTGGGTCGTGGTTTTCTGCCCCTCA
GCCTGCTCCTGAGAATGCTTATCAAGCTTATGCAGCACCTCAGCTGTTCCCAGTCTCCG
ACATAACCCAGAATCAACAGACTAACCAAGCCGGGGGAGAAGCACCTCAACCTGGAGAC
AATTCTACTGTTCAAACAGCAGCAGCAGTGGTGTTTGCTTGCCCCGGGGCTAACCAAGG
ACAACAGCTAGCAGACATTGGTGTTCCACAGCCTGCACCAGTGGCTGCCCCGGCACGAC
GCACACGGAAACCACAACAGCCAGAATCGCTGGAGGAATGCGATTCTGAACTAGAAATA
AAGCGATACAAGAATCGGGTGGCTTCCAGAAAATGCCGGGCCAAGTTTAAGCAACTGCT
GCAGCACTACCGTGAGGTGGCTGCTGCCAAATCATCTGAAAATGACAGGCTGCGCCTCC
TGTTGAAGCAGATGTGCCCAAGCCTGGATGTTGACTCCATTATCCCCCGGACACCAGAT
GTTTTACACGAGGATCTCTTAAATTTCTAA

B

AGA — ARGININE (R)

5 TGGACCCAAACTCGACTTCTGAAGATGTAAAATTTACACCTGACCCATACCAG 57

C

his-Zta K12R:

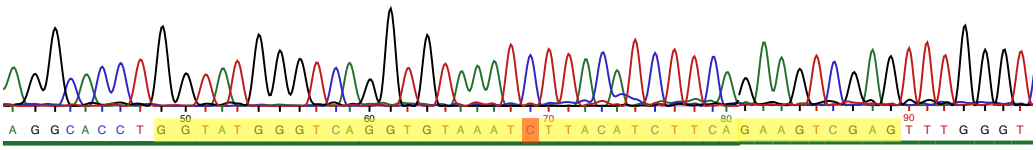


Figure 3.8 Zta sequence and the design process for the K12R mutant.
A. Zta ORF sequence. **B.** The mutagenic primer design. **C.** An example of the mutation validation chromatograph of his-Zta K12R. Key: - designed primer; - mutated region; - primers designed for sequencing.

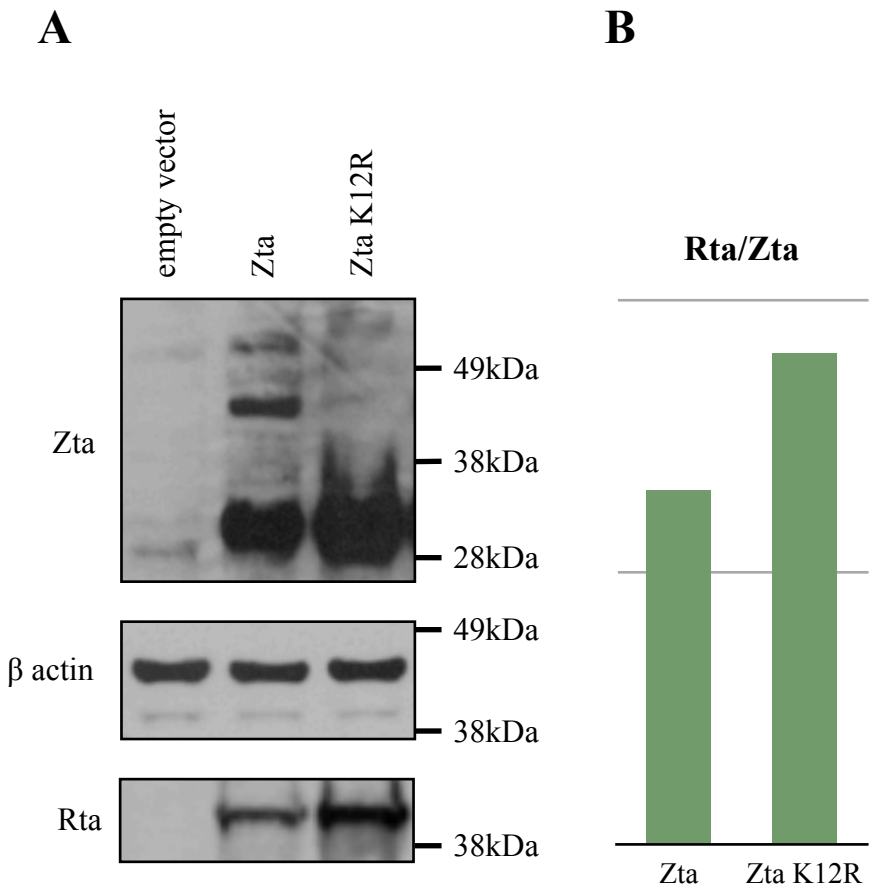


Figure 3.9 Role of Lysine 12 in post-translational modification of Zta.
A. HEK 293T EBV Z-KO cells were transfected with 1μg empty pBabe vector, wild type Zta or the K12R mutant (in a pBabe plasmid). The cells were then harvested 72 hours post-transfection and analysed by western blotting using anti β actin (loading control), anti-Rta and BZ1 (Zta) antibodies. **B.** Quantified Rta levels normalised to Zta protein levels.

3.2.5 VPK inhibits Zta's PTMs but not through the phosphorylation of Serine 209.

Previous findings by Asai et al. (2009) suggest that VPK could interact with Zta to modulate its function. To examine the ramifications of that interaction on Zta's post-translational modifications, a co-transfection of the 2 proteins into HEK 293T cells was performed. The data in Figure 3.10 highlights that VPK has a direct negative effect on Zta's PTMs, decreasing the abundance of all of the higher molecular forms: A, B and C. As Serine 209 of Zta is the proven site of VPK phosphorylation (Asai et al., 2009), two point mutants, Zta S209A and Zta S209D were generated (same method as for K12R outlined in Figure 3.8). The Zta S209A was designed to act as a phosphorylation incapable mutant, whereas Zta S209D was designed to mimic constitutive phosphorylation on residue 209. Both of these mutants however showed no effect on Zta's PTMs levels [Figure 3.11]. The Rta protein levels, on the other hand, showed an increase when the same amount of Zta S209A and Zta S209D was transfected compared to WT Zta [Figure 3.11]. These results indicate that phosphorylation of Serine 209 might have a negative effect on Zta's ability to transactivate the *BRLF1* gene.

3.2.6 Binding of Zta and VPK.

To test the assumption that VPK and Zta form a stable complex, first a lysis method using benzonase nuclease was devised, (as described in materials and methods) to efficiently extract proteins from HEK 293T cells. Benzonase is a nuclease enzyme that is capable of degrading nucleic acids in non-denaturing conditions. This allows extraction of nuclear proteins like Zta for further protein affinity chromatography, which was used to assess the protein-protein interaction of Zta and VPK.

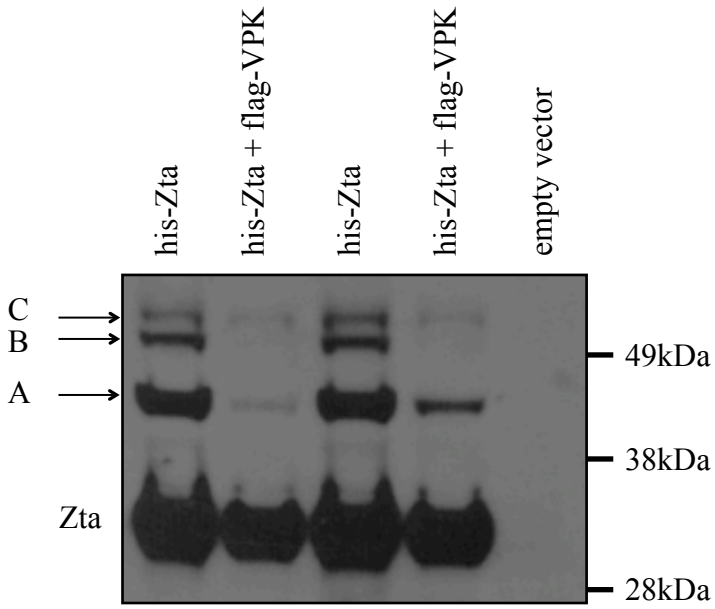


Figure 3.10 VPK inhibits Zta PTMs.
HEK 293T cells were transfected with 0.5µg his-Zta and with or without 0.5µg flag-VPK (with repeats), harvested 72h post-transfection and analysed by western blotting using BZ1 (Zta).

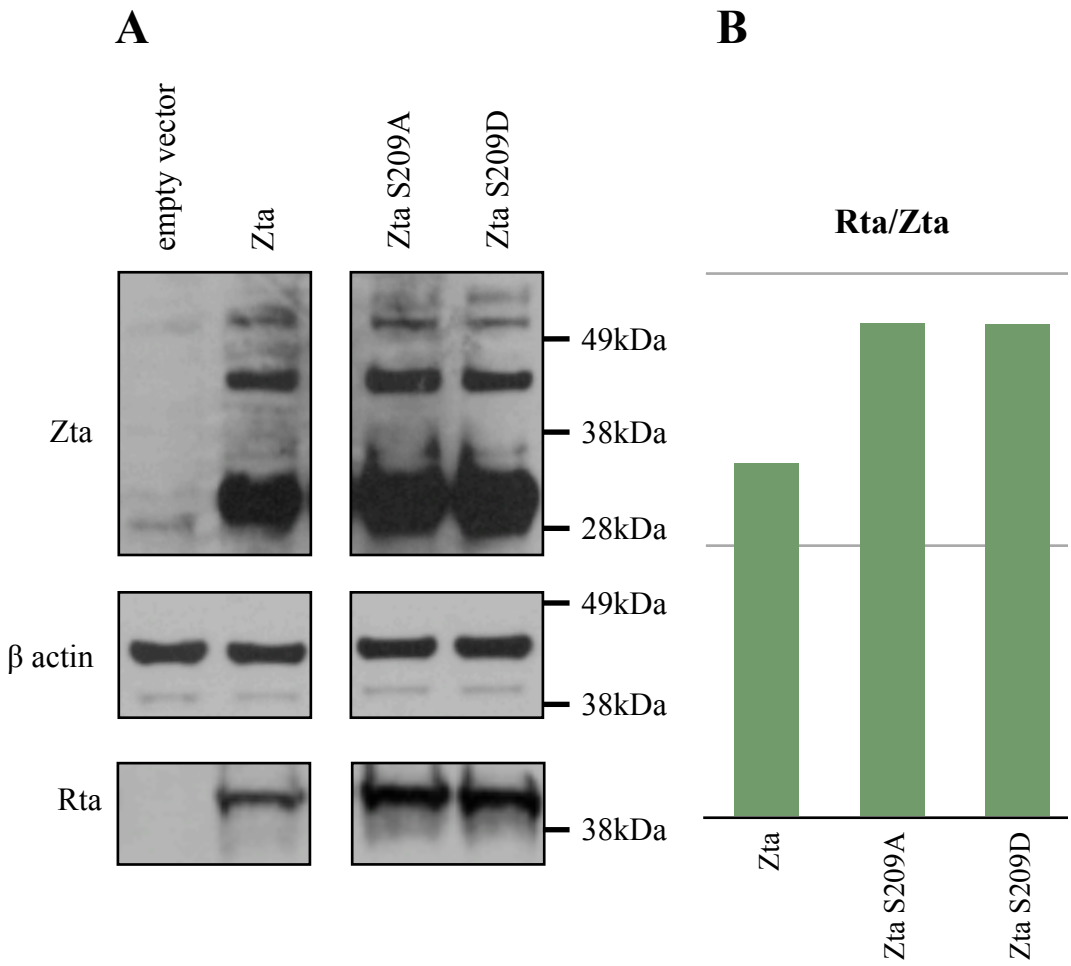


Figure 3.11 Phosphorylation on Serine 209 and Zta's PTMs.
A. HEK 293T EBV Z-KO cells were transfected with 1µg empty pBabe vector, wild type Zta, Zta S209A mutant and Zta S209D mutant (all in a pBabe plasmid). The cells were then harvested 72 hours post-transfection and analysed by western blotting using anti β actin (loading control), anti-Rta and BZ1 (Zta) antibodies. **B.** Quantified Rta levels normalised to Zta protein levels.

Data show [Figure 3.12] that the benzonase lysis was indeed an effective method for extracting both Zta and VPK from HEK 293T cells. Therefore, a Flag pull-down experiment was performed with Zta and all the designed point mutants (K12R, S209A and S209D). Unexpectedly, Zta did not co-elute with Flag-VPK [Figure 3.13] indicating the absence of protein-protein interaction between the Zta and VPK. A reverse pull-down using Nickel beads to bind histidine-tagged Zta was also attempted and showed similar results (data not shown).

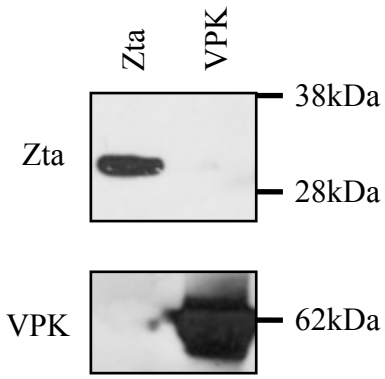


Figure 3.12 Benzonase lysis.
HEK 293T cells were transfected with Zta or flag-VPK (1µg in total) and harvested 72h post-transfection using benzonase lysis method. The samples were then analysed by western blotting using BZ1 (Zta) and anti-Flag (VPK) antibodies.

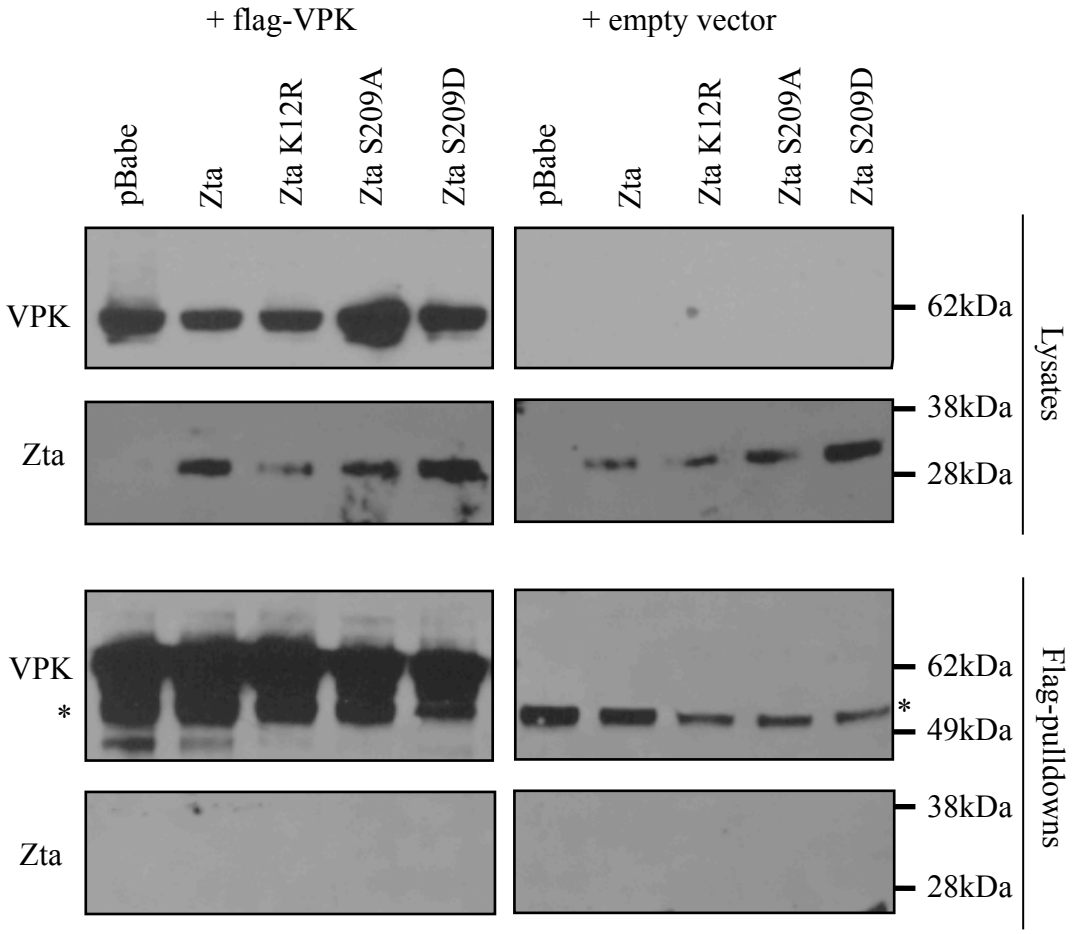


Figure 3.13 Flag pulldowns.
HEK 293T cells were transfected with the corresponding plasmids, harvested 72h post-transfection and lysed using benzonase method. An aliquot of the total benzonase lysis was mixed with 2xPSB, loaded onto a gel and analysed by western blotting (Lysates). The remaining samples were put though a flag pull-down and analysed by western blotting. Anti-flag and BZ1 antibodies were used to visualise the result. Marked by asterisks are the heavy chains from the pull-down antibodies. N.B. The amount of flag-pulldowns loaded correspond to 5x of the lysates loaded.

3.3 Chapter discussion.

Over the past decade EBV's lytic cycle involvement in the ATM mediated part of the DDR has become more apparent. Both Zta and VPK have been implicated in modulating the proteins of the DDR signal transduction pathway. Kudoh et al. (2005) noted that upon reactivation of the lytic cycle in B95-8 cells with an inducible Zta system, the ATM, p53, Chk2 and H2AX proteins become activated and an S-phase like cellular environment is established (Kudoh et al., 2005). It is possible that ATM is activated by newly replicated viral genomes that might be recognised as double strand breaks. Nevertheless, Li et al. (2011) showed that VPK phosphorylates and switches on the acetyltransferase TIP60, which implies an active induction of the DDR by EBV. The virus might benefit from upstream effects of DDR like acetylation of histones and phosphorylation of H2AX at oriLyt and viral promoters (Li et al., 2011). Moreover, Zta and p53 co-localise in viral replication compartments in the nucleus. However, the p53 transcription targets, MDM2 and p21 exhibit a decreased protein level expression during the lytic cycle (Kudoh et al., 2005). Sato et al. (2009a) showed that inhibition of p53 is directly mediated by Zta. By acting as an adaptor of the ECS Ubiquitin ligase, Zta can target p53 for proteasomal degradation. Furthermore, the DDR induced phosphorylated form of p53 is able to bind Zta with a higher affinity. Thus, Zta can inhibit deleterious effects downstream of the DDR mediated through p53 (Sato et al., 2009a).

Since the correlation of VPK expression and H2AX phosphorylation was observed only in B-cells (Li et al., 2011) or murine fibroblasts (Tarakanova et al., 2007), the present study utilised HEK 293T cells to assess the effects VPK and Zta on H2AX phosphorylation levels. The data showed that neither VPK, nor additional Zta

supplementation, affected the γH2AX levels in the absence of other viral proteins in HEK 293T cells. This observation suggests that other factors are involved in VPK mediated phosphorylation of H2AX. It is therefore important to consider the cellular background when choosing a cell line to study these proteins, as the results can be cell type dependent. However, to confirm these results further investigations are needed that should include controls for biological activity of VPK kinase. This can be undertaken by utilising a known VPK substrate that can be immunobloted on phosphorylated residues. In addition, a control for H2AX phosphorylation would also be necessary. This could be achieved by DNA damage inducing agents like etoposide.

Zta is the master lytic regulator of EBV that can act as a transcription factor, origin of replication binding protein, or can directly interact with other proteins to elicit an effect in the infected cells. Because of its many roles and importance, fine-tuning Zta's function is essential for EBV. One way this is achieved is through post-translational modifications of the protein. Adamson and Kenney (2001) reported that Zta can be modified by SUMO conjugation. In the present study a number of Zta expressing epithelial and B-cells were examined for the higher molecular weight variants of Zta. At least three different forms, A, B and C, in addition to the unmodified protein were observed. The A form is most likely a monoSUMOylated Zta, whereas the B and C forms are polySUMOylated versions. The presented data suggests that the higher molecular weight forms of Zta were not dependent on other viral proteins and were not cell type specific. However, it was noticed that B-cell lines had generally lower levels of modifications compared to the Zta in epithelial cells (with exception of the C form in Akata cells). To strengthen these conclusions a titration of Zta in the studied cell lines would

be necessary, and together with a loading control (e.g. actin), as well as the use of a single gel for all samples would help to better visualise and compare the higher molecular weight forms of Zta. Nevertheless, a study by Murata et al. (2010) published during this project confirm the presented findings, and together the results suggest that the small protein PTMs attached to Zta are at lower abundance in B cells.

The possibility that Zta might be Ubiquitylated has not been questioned in the literature. Because one of the major roles of Ubiquitylation is signalling for proteasomal degradation, here it was tested whether Zta could be targeted for proteolysis. The results showed that Zta levels did not increase upon inhibition of the proteasome in human epithelial cells. This suggests that Zta's turnover is not controlled by Ubiquitin mediated proteasomal degradation. To exhaustively prove that Zta is only SUMOylated and not Ubiquitylated further investigations are needed. Tagged versions of SUMO and Ubiquitin proteins could be co-transfected with Zta and subjected to immunoprecipitation or affinity chromatography with subsequent western blot analysis alongside a wild type Zta with observable higher molecular weight forms.

The involvement of the extreme carboxy-terminus of Zta, as well as the Lysine 12 residue on the SUMOylation of Zta were also investigated in this chapter. The results show that Lysine 12 is required for SUMOylation of Zta. The three C-terminal amino acids of Zta however, do not play a role in this process. In addition to the effects on Zta PTMs, the impact of the mutants on the ability to activate Rta was indirectly observed. Rta protein levels increased when Lysine 12 or the carboxy terminus of Zta was mutated, suggesting that these residues have an inhibitory effect on Zta's ability to transac-

tivate the immediate early *BRLF1* gene. In their publication, Bailey et al. (2009) also detected a moderate increase in activation of the *BMRF1* gene with the Zta 243ter mutation. While Adamson (2005) found a significant reduction in the Rta promoter activity when Zta and SUMO were co-expressed in an *in vitro* assay.

To investigate the role of VPK in Zta PTMs, Serine 209 mutants of Zta were generated and protein affinity chromatography was attempted. Unfortunately, neither the VPK Flag nor the 6xhistidine tagged Zta Nickel affinity (data not shown) pull-downs exhibited any co-elution of Zta and VPK. Even though Asai et al. (2006; 2009) showed that VPK and Zta can interact with a resultant phosphorylation on Serine 209, other genome wide studies did not reproduce these results. An *in vitro* protein array kinase assay did not identify Zta as a target of VPK (Zhu et al., 2009). Moreover, an EBV protein-protein interaction study using a yeast two-hybrid system, could not identify any binding between VPK and Zta (Calderwood et al., 2007). All of these observations could be explained by the fact that the interaction (with the resulting phosphorylation) of Zta and VPK is transient and might be affected by the cellular environment. Nonetheless, the co-transfection of Zta and VPK in HEK 293T cells showed that VPK can markedly decrease the levels of SUMOylated Zta. This inhibition partly explains the higher abundance of A, B and C forms of Zta in cells without the EBV infection (e.g. HeLa cells). The decrease in SUMO conjugated Zta could be mediated through three possible mechanisms: A. VPK phosphorylates intermediate factors, which ultimately results in de-SUMOylation of Zta; B. Direct VPK phosphorylation on Serine 209 of Zta, acts as a signal for de-SUMOylating enzymes; and C. Phosphorylation dependent interaction of Zta and VPK recruits proteins that target Zta for SUMO de-conjugation [Fig-

ure 3.14]. These models were tested with the help of the designed phosphorylation dead S209A and phosphomimic S209D Zta mutants. The data revealed that neither of these variants could affect the levels of the higher molecular forms of Zta, implying that phosphorylation on Serine 209 does not play a role in Zta SUMOylation. Therefore VPK mediated inhibition of SUMO-Zta must occur though some as yet unknown, VPK regulated intermediaries [Figure 3.14 (A)]. As this study was progressing, the publications of Hagemeier el al. (2010) and Murata et al. (2010) were released, the results of which coincided with the ones described above. They found that a decrease in Zta SUMOylation is attributed to VPK, but is independent of the phosphorylation on Serine 209; whilst Hagemeier et al. (2010) also saw an increase in Rta protein levels whenLysine 12 was mutated, validating the findings presented in this study. Their group showed that all three major forms of SUMO (1, 2 and 3) are covalently attached to Zta. SUMO was linked to a general decrease in Zta's ability to activate viral promoters, but had no significant effects on the production of new virion particles (Hagemeier et al., 2010). Murata et al. (2010) investigated further the SUMO inhibition of Zta's transcriptional capacity. They linked this phenomenon to the ability of SUMO-Zta to bind histone deacetylase 3 (HDAC3). Using chromatin immunoprecipitation they showed that HDAC3 is recruited by SUMO-Zta to the ZRE containing regions of the viral genome (Murata et al., 2010).

Together with the published results, the data herein shows that VPK can affect Zta in two distinct and opposing ways. Firstly, independent from the phosphorylation of S209, VPK can decrease the levels of Zta's SUMOylation, thus elevating its

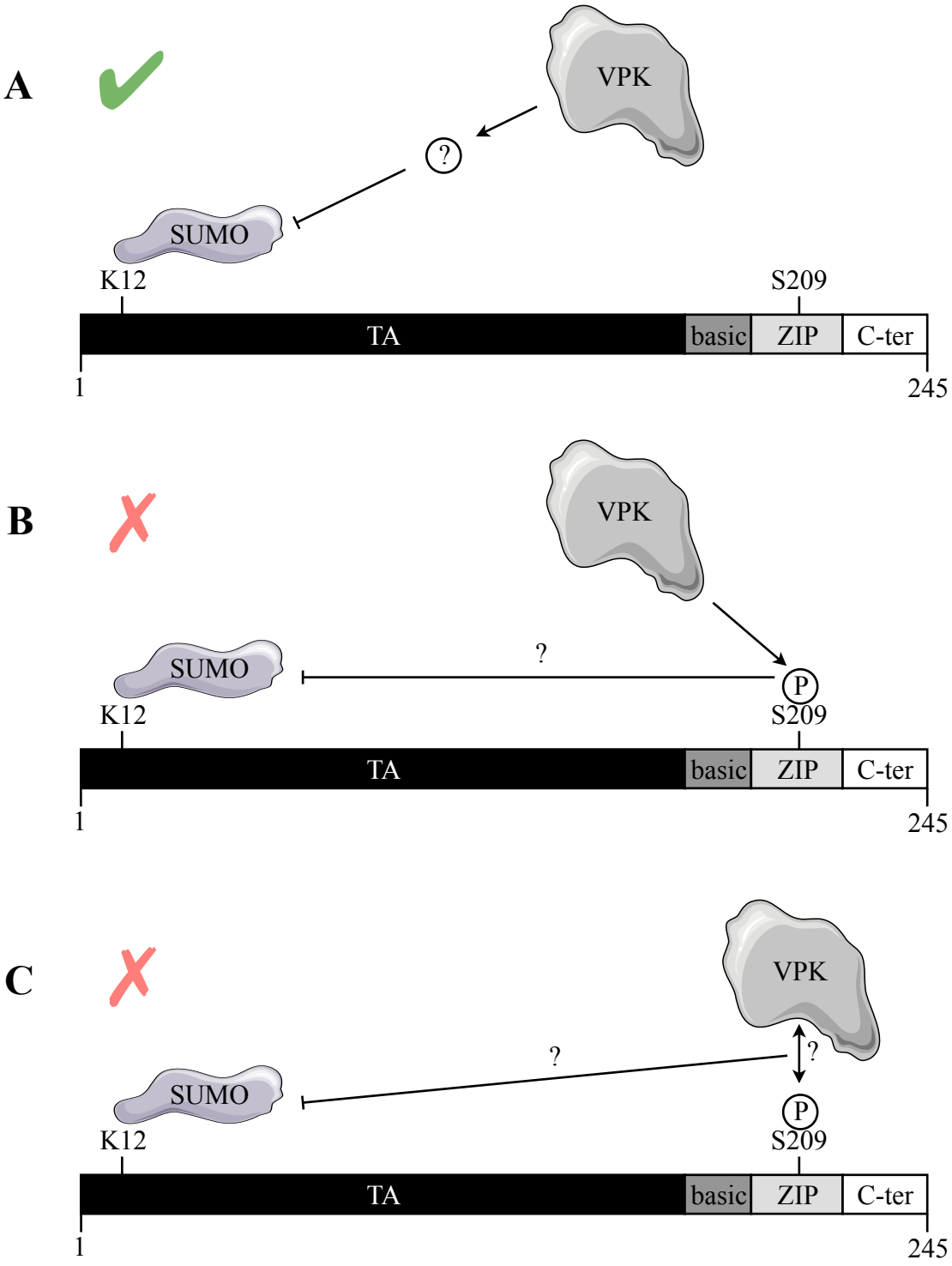
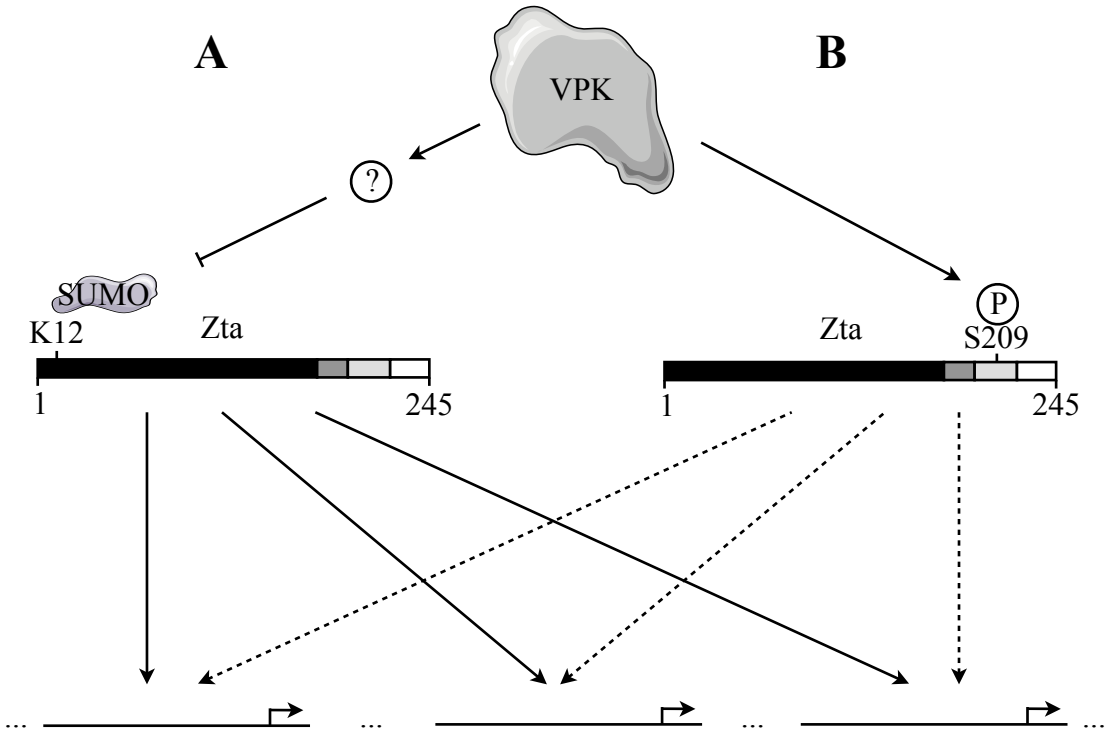


Figure 3.14 Possible mechanisms of SUMO-Zta inhibition by VPK.
3 possible mechanisms of action of VPK on PTM inhibition of Zta. **A.** VPK dependent de-SUMOylation of Zta happen via interaction and phosphorylation of intermediate proteins. **B.** Phosphorylation on S209 of Zta is a signal for de-SUMOylation. **C.** Phosphorylation dependent VPK and Zta binding signals for the removal of the SUMO moiety.

transactivation potential. Secondly, VPK can directly phosphorylate Zta on Serine 209, hampering its transcription ability [Figure 3.15]. This dual mechanism of action could prove to be beneficial for EBV, providing a strategy by which the virus can regulate lytic transcription. Considering that the ChIP data indicates that Zta can also bind to the *BGLF4* promoter (data not shown), a potential feedback loop might take place. As the lytic cycle progresses and VPK starts to be expressed, low levels of the kinase might be beneficial for Zta's transactivation, since the multistep effect of SUMO inhibition through various intermediaries has the potential to be amplified. Once the protein concentration of VPK reaches a critical level however, the direct phosphorylation of Zta on Serine 209 would start to have a noticeable impact, counteracting the positive effects of SUMO inhibition. This would cause a decrease in Zta's transcriptional ability and would stabilise the expression various lytic proteins, including VPK and Zta. Thus, through the positive and negative feedback loops an equilibrium could be established.



Gene promoters controlled by Zta

Figure 3.15 VPK's effects on Zta.
VPK can affect Zta in two distinct ways. **A.** VPK can inhibit SUMOylation of Zta on Lysine 12 through an unknown intermediate, which subsequently causes an increase in Zta's transactivation. **B.** VPK can directly phosphorylate Zta on Serine 209 to decrease its transcription ability.

4. Comparative analysis of Zta's contribution to promoter activation and repression.

4.1 Introduction.

Zta is known to have various functions in the cell. Among the most significant and earliest discovered, is the ability of Zta to act as a transcription factor (Chevallier-Greco et al., 1986). One of the viral genes that has a high reliance on Zta for its transcription, is *BHLF1* (Hardwick et al., 1988; Lieberman et al., 1989). The *BHLF1* gene is highlighted by containing a region of NotI repeats, and is of one the most abundant EBV RNA species found in lytically induced cells (Hummel and Kieff, 1982; Jeang and Hayward, 1983; Jones and Griffin, 1983). Because of these properties, *BHLF1* RNA has been used as a marker of lytic infection, detected by in-situ hybridisation (Drut et al., 1994; Montone et al., 1992; Ryon et al., 1993). Information about the BHLF1 protein is scarce and apart from the early studies, much still remains unknown about the gene product. All that is known about the protein, is that it contains tandem polypeptide repeats (due *Not*I repeats in its ORF), has a possible nucleolar localisation, and a high affinity for ssDNA (single stranded DNA) (Lieberman et al., 1989; Nuebling and Mueller-Lantzsch, 1989). The *BHLF1* promoter, on the other hand, has been studied in more detail, possibly because it is found adjacent to the OriLyt and is essential for viral lytic replication (Schepers et al., 1993b). The four ZREs found in the *BHLF1* promoter [Figure 4.1 (A)], which are the binding sites for Zta, were shown to be critical for both EBV replication and *BHLF1* transcription (Schepers et al., 1993a, 1996). Recently, Rennekamp and Lieberman elucidated the processes involved further. They demonstrated that the RNA transcript of *BHLF1* binds to the NotI repeats on the DNA, creating a so

called R-loop, which is essential for the recruitment of the EBV ssDNA Binding Protein (BALF2) to the OriLyt (Rennekamp and Lieberman, 2011). They also confirmed an interaction between BALF2 and Zta, which was previously shown to occur through the EBV helicase-primase (BBLF4-BBLF2/3-BSLF1) complex, all of which is essential for OriLyt replication (Gao et al., 1998; Rennekamp and Lieberman, 2011). Furthermore, viral DNA polymerase processivity factor (BMRF1), immediate-early transcription factor Rta, and cellular Ku80 have been shown to interact with Zta and synergistically enhance transcription of *BHLF1* (Chen et al., 2011; Chen et al., 2005; Zhang et al., 1996). Additionally, Bergbauer et al. concluded that the methylation status of the CpG motifs in the *BHLF1* promoter had no significant effect on the ability of Zta to bind and activate the *BHLF1* promoter (Bergbauer et al., 2010).

Another gene that has been shown to be regulated by Zta, is the cellular *CIITA* (Li et al., 2009). The CIITA protein encoded by this gene is often referred to as the master activator of the MHC class II expression, which is central in antigen presentation by specialised cells in acquired immunity (Steimle et al., 1993; Steimle et al., 1994). Due to its role, *CIITA* has a highly complex and regulated promoter region. Four different promoters have been identified, that control *CIITA* gene expression: pI, pII, pIII and pIV (Muhlethaler-Mottet et al., 1997). Except for the pII driven transcript, the function of which still remains unknown, pI, pIII and pIV, all produce different CIITA isoforms, that vary only at the amine terminus (Muhlethaler-Mottet et al., 1997). Promoter I of *CIITA* is constitutively active in dendritic cells and INF-γ induced macrophages (Muhlethaler-Mottet et al., 1997; Waldburger et al., 2001). Promoter IV is essential for the expression of MHC class II in non-hematopoietic cells, where it is either expressed

constitutively (thymic epithelial cells), or induced by INF-γ (astrocytes, epithelial cells, etc.) (Waldburger et al., 2003; Waldburger et al., 2001). T cells and B cells on the other hand, depend on pIII for the expression of CIITA and consequently their antigen presentation ability (Holling et al., 2002; Muhlethaler-Mottet et al., 1997). Therefore, it is of no surprise, that pIII of *CIITA* is the site of regulation by Zta. Immune evasion is an important strategy required for viral survival and the ability to control CIITA expression would be highly advantageous for EBV. Indeed, Zta is able to repress the *CIITA* promoter by binding to the TSS proximal ZRE (Li et al., 2009). This finding is somewhat controversial, as direct DNA binding by Zta usually confers activation of a promoter and repression is achieved via competition for the binding sites of other transcription factors, which does not seem to be the case with *CIITA* (Li et al., 2009; Sato et al., 1992). Nonetheless, it would be expected that distinct parts of the Zta protein would be responsible for promoter activation as opposed to promoter repression.

The aim of this chapter is to investigate the differences in Zta's protein coding region in respect to its ability to repress and activate gene expression, using *BHLF1* and *CIITA* promoters as a model.

4.2 Results.

4.2.1 BHLF1 and CIITA as model promoters.

BHLF1 has one of the most extensively studied promoters among EBV genes. Because it is also highly regulated by Zta, and greatly activated by direct Zta binding to DNA, it was decided to use it as a model promoter. Figure 4.1 (A) shows a diagram of the promoter region that was used in the Luciferase assay experiments. The cloned promoter contains the TSS (indicated by the arrow) and the four ZREs that are essential for transcriptional activation and DNA binding by Zta, as well as the TATA and CCAAT boxes (Schepers et al., 1993a, 1996). The construct, subcloned into a pCpGL backbone (designed on the basis of pGL3 plasmid by Klug and Rehli (2006)), was kindly provided by professor Wolfgang Hammerschmidt and was shown to be activated by Zta in HEK 293T cells, in Bergbauer's et al. (2010) research. Indeed, the Luciferase assay data [Figure 4.2 (A)] show that the *BHLF1* promoter was activated up to ~150 times in HEK 293T cells when his-Zta was co-transfected, providing a suitable assay for further investigations. A number of ways to control the transfection efficiency of the Luciferase assays exist on the market, including pRL-SV40, pRL-TK and pRL-CMV vectors (according to Promega manual). However, the *in silico* investigations of the nucleotide sequence of these vectors revealed that all of them contained four to five ZREs in the promoter region, which could potentially bind Zta. Indeed, co-transfection trials of Zta and pRL-TK (most commonly used control) performed in our laboratory revealed that pRL-TK expression levels were affected by Zta (data not shown). To compensate for the lost control it was decided to use Bradford assays in conjunction with western blotting of both actin and Zta (or its modified versions) in each Luciferase assay performed.

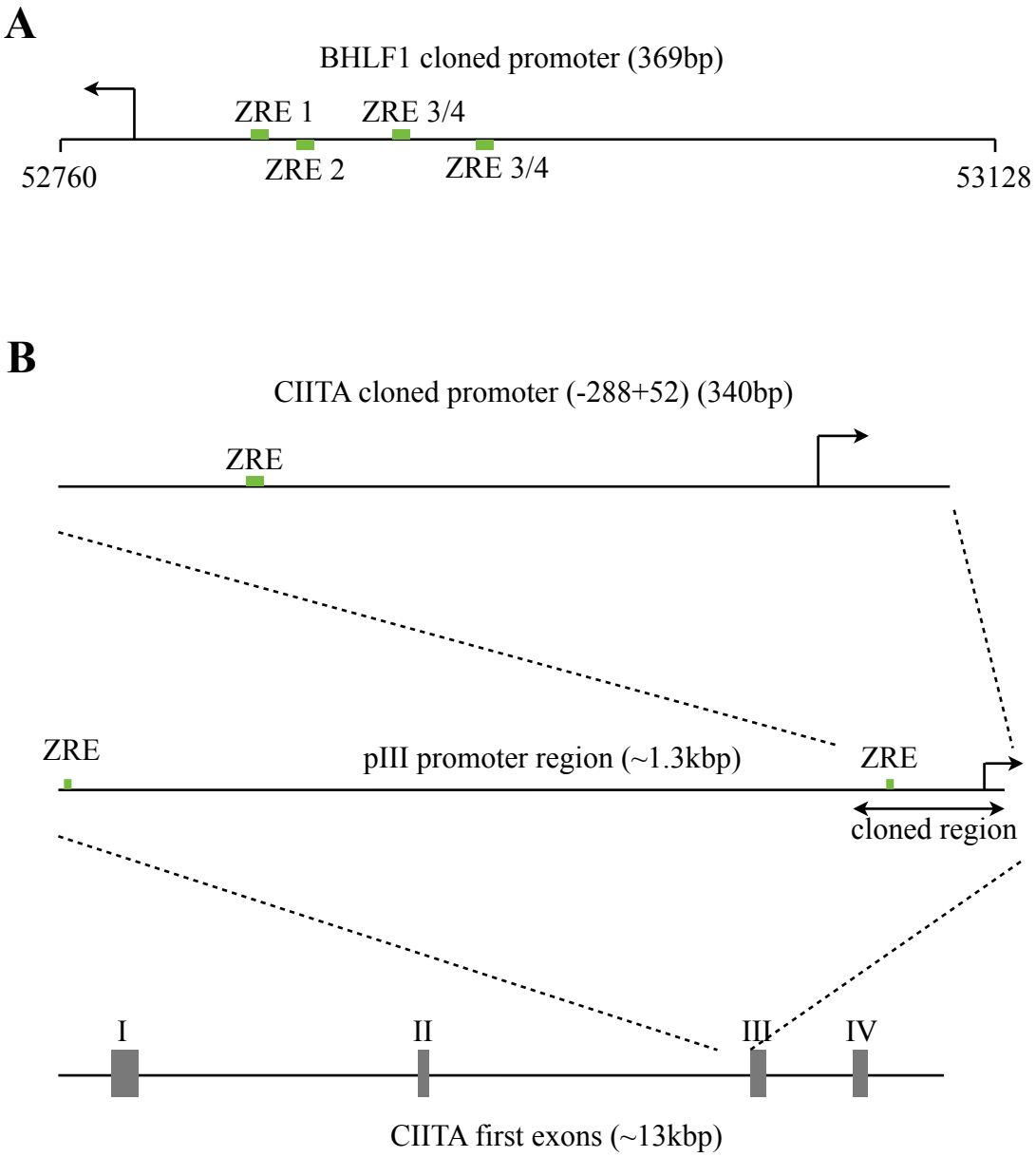


Figure 4.1 Graphical representation of the cloned promoters.
A. Graphical representation of the BHLF1 promoter region cloned and used for the Luciferase reporter assay system. Position of the four ZREs is shown in green (NB. The two ZRE3/4 have identical sequences), as well as the position of the TSS, indicated by the arrow. The location coordinates in the EBV B95-8 strain is also shown (52760-53128) (Bergbauer et al., 2010). **B.** Schematic representation of CIITA's possible first exons (numbered grey boxes) and of the zoomed in pIII promoter region situated immediately in front of exon III. Two ZREs found in the pIII promoter are shown, as well as the TSS and the cloned region used for the Luciferase reporter assay system (Li et al., 2009; Muhlethaler-Mottet et al., 1997).

CIITA on the other hand, is repressed by Zta (Li et al., 2009). A promoter region similar to the one used by Li et al. (-288+52, compared to the published -288+44), containing the TSS and the proximal ZRE [Figure 4.1 (B)] was designed and subcloned into a pGL3 enhancer vector using KpnI and HindIII restriction sites. Unlike pGL3 basic, pGL3 enhancer contains an SV40 enhancer region downstream of the Luciferase gene. Subcloning into this backbone provides a more sensitive system that exhibits a higher basal promoter activity (according to the manufacturers manual). The Luciferase data [Figure 4.2 (B)] show a substantial level of repression of the *CIITA* promoter in Raji cells, when his-Zta rather than the empty vector (pcDNA3) were co-transfected. The repression was not dependent on the poly-histidine tag, as the untagged Zta (in a pBabe backbone) also repressed the promoter to a similar extent when adjusted to Zta protein levels (data not shown). As the pBabe-Zta system exhibited a lower protein expression, compared to his-Zta in pcDNA3, the latter was chosen as a more suitable option for further assays. In addition, the repression of the *CIITA* pIII promoter construct was also tested in the more viable and manageable HEK 293T cells. However, because pIII of *CIITA* is most likely lymphoid specific, HEK 293T epithelial cells are not expected to express the pIII. Indeed, the basal promoter activity of the construct was low and no Zta repression was observed in HEK 293T cells either (data not shown).

4.2.3 Repression and activation by Zta is not dependent on viral co-factors.

Viral co-factors can play an important role in the process of transcriptional regulation by Zta (Chen et al., 2005; Zhang et al., 1996). To test if repression and activation by Zta is dependent on viral factors, an EBV free Akata cell line, AK31, was used for the Luciferase assay system. Data in Figure 4.3 show that even in the absence of other EBV

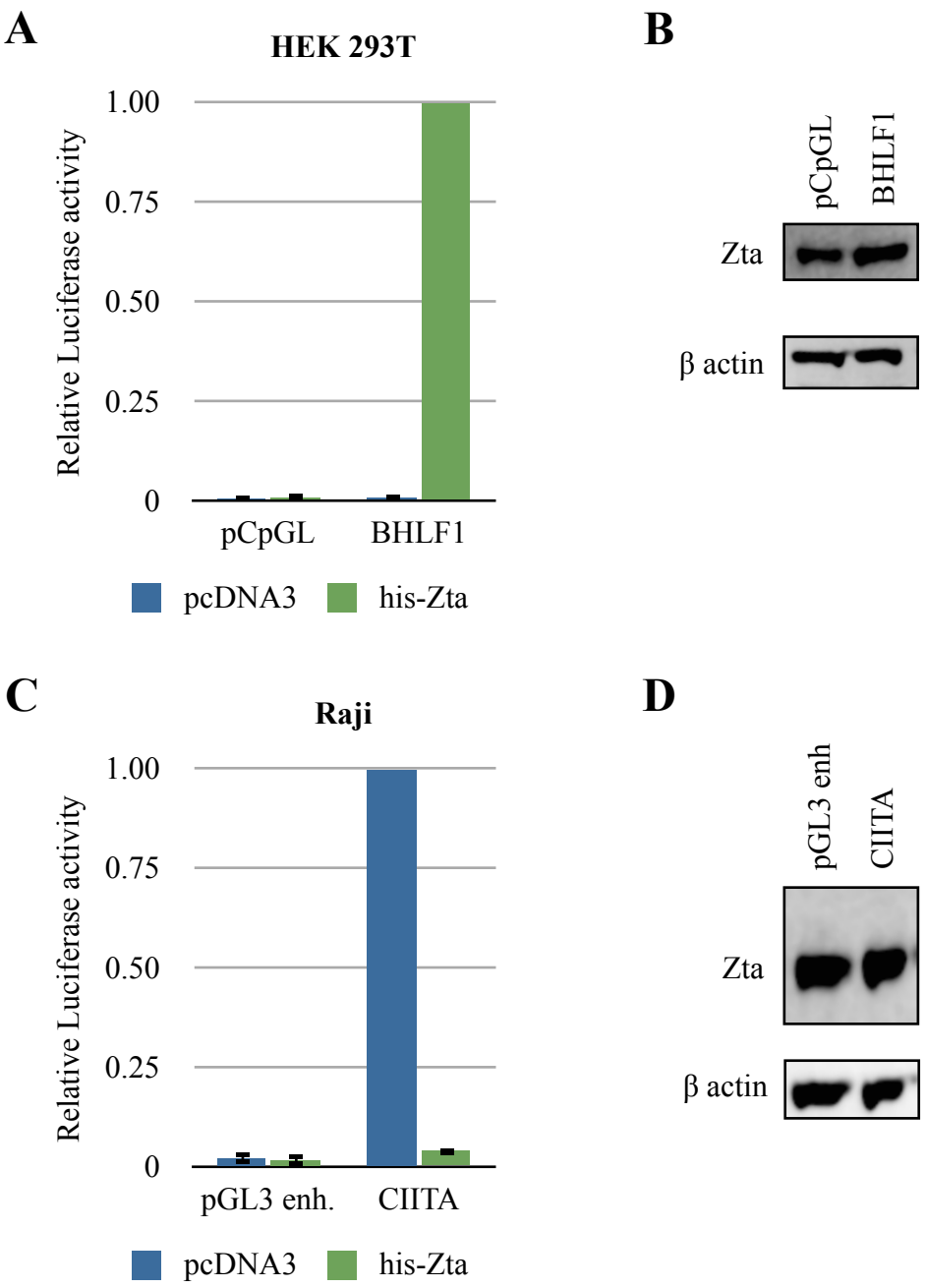


Figure 4.2 Zta represses CIITA and activates BHLF1 promoters.
A. Luciferase assay data showing the effects of his-Zta on the empty vector (pCpGL) or the BHLF1 promoter in HEK 293T cells. The values were adjusted to total protein concentration and normalised to his-Zta + BHLF1. Error bars indicate standard deviation; data from two biological replicates. **B.** Western blot analysis of the HEK 293T cellular extracts using BZ1 (Zta) and anti-β actin antibodies. **C.** Luciferase assay data showing the effects of his-Zta on the empty vector (pGL3 enh.) or the CIITA promoter in Raji cells. The values were adjusted to total protein concentration and normalised to pcDNA3 + CIITA. Error bars indicate standard deviation; data from three biological replicates. **D.** Western blot analysis of the Raji cellular extracts using BZ1 (Zta) and anti-β actin antibodies.

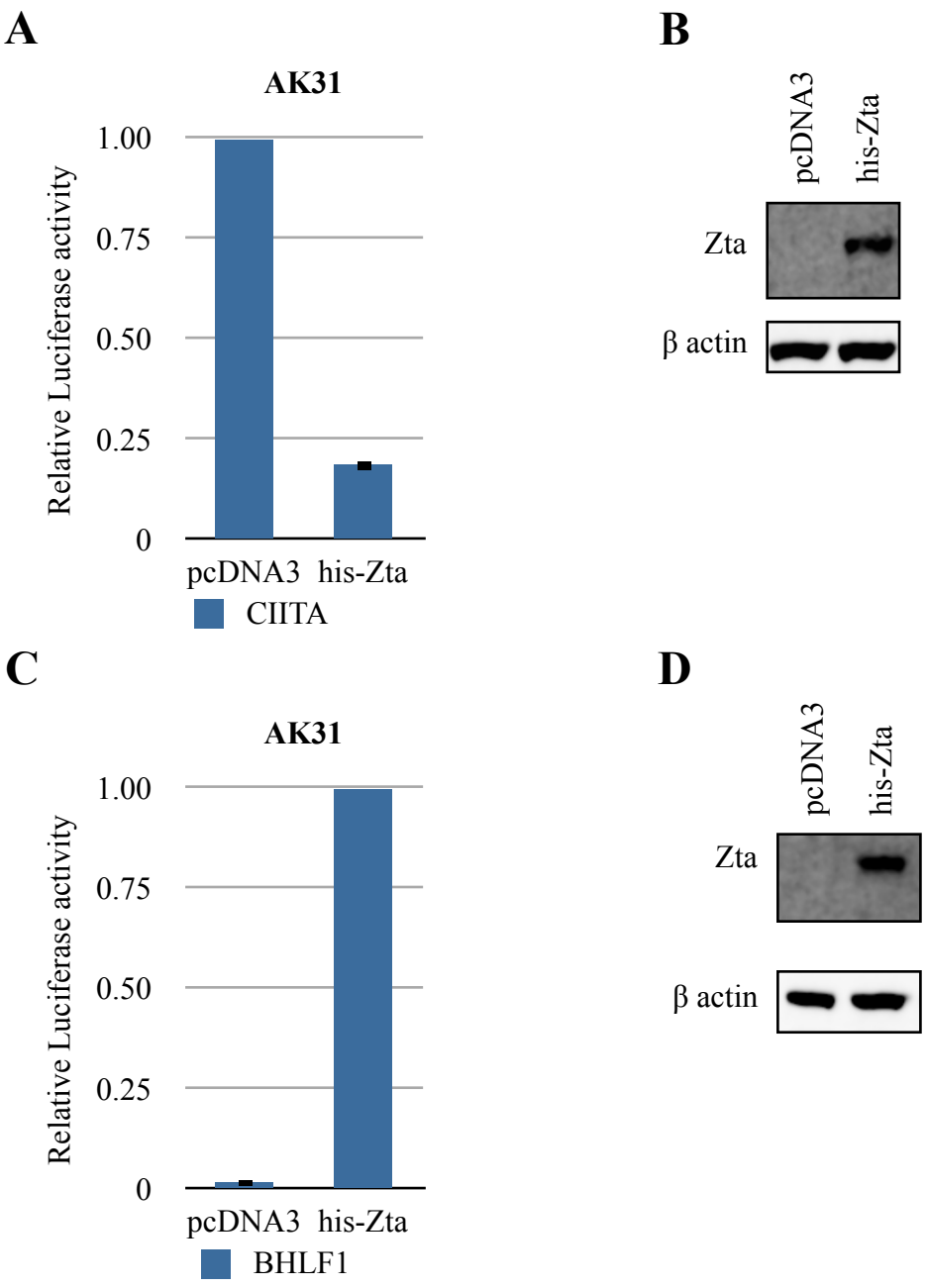


Figure 4.3 Repression and activation by Zta does not require viral proteins. AK31 cells were transfected with his-Zta or empty vector (pcDNA3) and the CIITA or BHLF1 promoters and processed according to Luciferase assay protocol. **A.** Luciferase assay data showing the effects of his-Zta on the CIITA promoter. The values were adjusted to total protein concentration and normalised to pcDNA3. Error bars indicate standard deviation; data from two biological replicates. **B.** Western blot analysis of the CIITA promoter transfections using BZ1 (Zta) and anti-β actin antibodies. **C.** Luciferase assay data showing the effects of his-Zta on the CIITA promoter. The values were adjusted to total protein concentration and normalised to his-Zta. Error bars indicate standard deviation; data from two biological replicates. **D.** Western blot analysis of the BHLF1 promoter transfections using BZ1 (Zta) and anti-β actin antibodies.

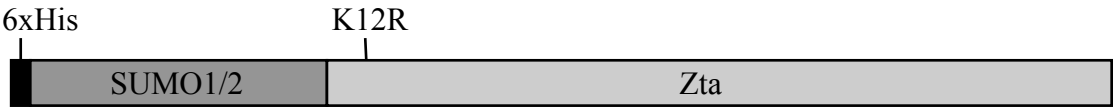


Figure 4.4 Graphical representation of SUMO-fusion protein primary structure. Schematic representation of the SUMO-Zta fusion proteins made. A 6xhistidine tag followed by either SUMO-1 or SUMO-2 C-ter truncated (-6 and -4 aa respectively) proteins and the Zta protein with Lysine 12 mutated that were created is shown.

proteins and transcripts, his-Zta was able to both repress the pIII of *CIITA* and activate the *BHLF1* promoters. These results suggest that both positive and negative modulation of gene expression of these promoters by Zta is independent of the lytic or latent EBV backgrounds.

4.2.4 SUMO-Zta fusion proteins.

SUMO conjugation to Zta is known to inhibit its transcriptional activity and a Zta Lysine 12 mutant defective in SUMOylation, was shown to transactivate at higher levels than the wild type protein in HeLa cells (Adamson, 2005; Hagemeier et al., 2010). In order to further study the effects of the SUMOylation on Zta's transcriptional ability, two fusion proteins, his-SUMO1-Zta and his-SUMO2-Zta were designed. The fusion with SUMO3 isoform that was also reported to interact with Zta was not made, as it has high homology and very similar functions with the SUMO2 protein (Saitoh and Hinchey, 2000). Since the Lysine 12 at which the conjugation with SUMO was shown to occur, is located very close to the amino terminus of Zta, it was decided to place the ORF of SUMO proteins in front of the Zta's sequence. The constructs were designed with a new start codon and 6 histidines at the N-terminus followed by either SUMO1 or SUMO2 and Zta ORFs [Figure 4.4]. SUMO1 and SUMO2 sequences had the stop

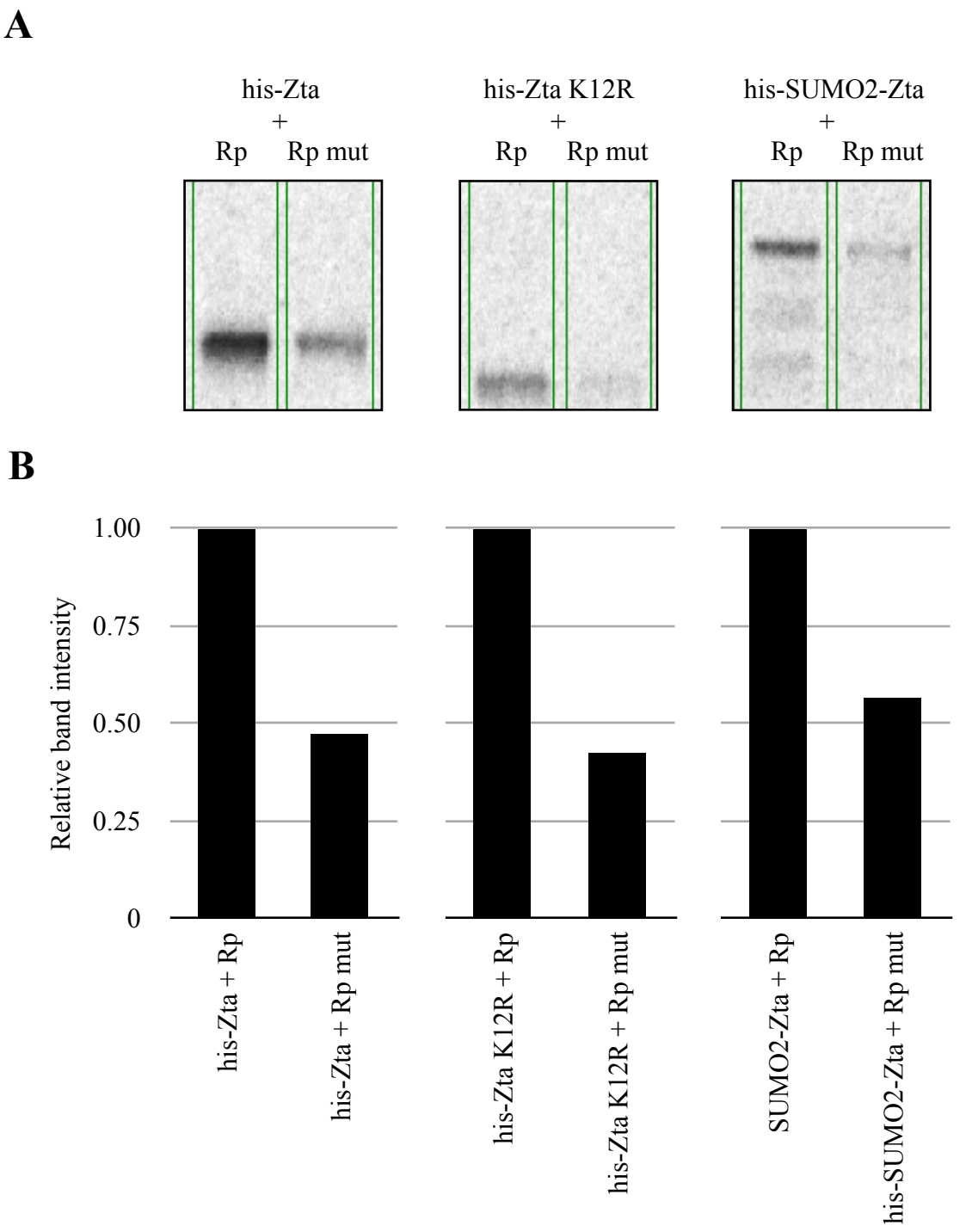


Figure 4.5 SUMO fusion protein and Lysine 12 mutant can bind DNA *in vitro*. Corresponding proteins were expressed in the presence of radioactively labeled amino acids using rabbit reticulocyte lysate (RRL) system. RRL were quantified and equivalent amount of each protein extract was incubated with DNA oligos (Rp and Rp mut) in vitro. **A.** Radiograph of the samples after the binding assay. **B.** Quantification of each radiographic band. Values were normalised protein + Rp promoter levels.

codon removed and were made shorter by 6 and 4 carboxy-terminal amino acids respectively. This was done to eliminate the di-glycine motif that is used by SUMO specific proteases for deconjugation of the SUMO PTM (Gong et al., 2000; Li and Hochstrasser, 1999). The Zta sequence was also modified by the introduction of K12R mutation, to avoid multiSUMOylation of Zta [Figure 4.4]. Finally, the full sequences were ordered from Eurofins MWG, subcloned into a pcDNA3 expression vector using the BamHI and EcoRI restriction sites. The resulting vectors were sequenced for errors and amplified in *E.coli* according to the protocol.

4.2.5 SUMO-Zta fusion and Zta K12R proteins can bind DNA *in vitro*.

Zta mediated transcriptional control of both *BHLF1* and *CIITA* has been reported to be dependent on direct DNA binding to the ZRE sequences (Li et al., 2009; Schepers et al., 1993a, 1996). To examine this crucial functionality in the SUMO Zta fusion and the earlier mentioned Zta K12R proteins, an *in vitro* assay was carried out. First, the mRNA of interest was amplified *in vitro* from the pcDNA3 containing the protein constructs and purified using a Promega kit. Each mRNA species was introduced into a rabbit reticulocyte lysate (RRL) system and translated in the presence of radioactively labelled amino acids. The RRLs were then incubated with Dyna beads coupled with DNA oligonucleotides, known to bind (Rp promoter) or not to bind (Rp mut) Zta. Figure 4.5 (A) shows the western blot radiographic analysis of the resuspended Dyna beads. The quantified and normalised data [Figure 4.5 (B)] show that all the tested proteins had similar *in vitro* DNA binding compared to the background binding to the negative control (Rp mut), indicating that the amino terminal fusion of SUMO did not impair Zta in its ability to bind DNA.

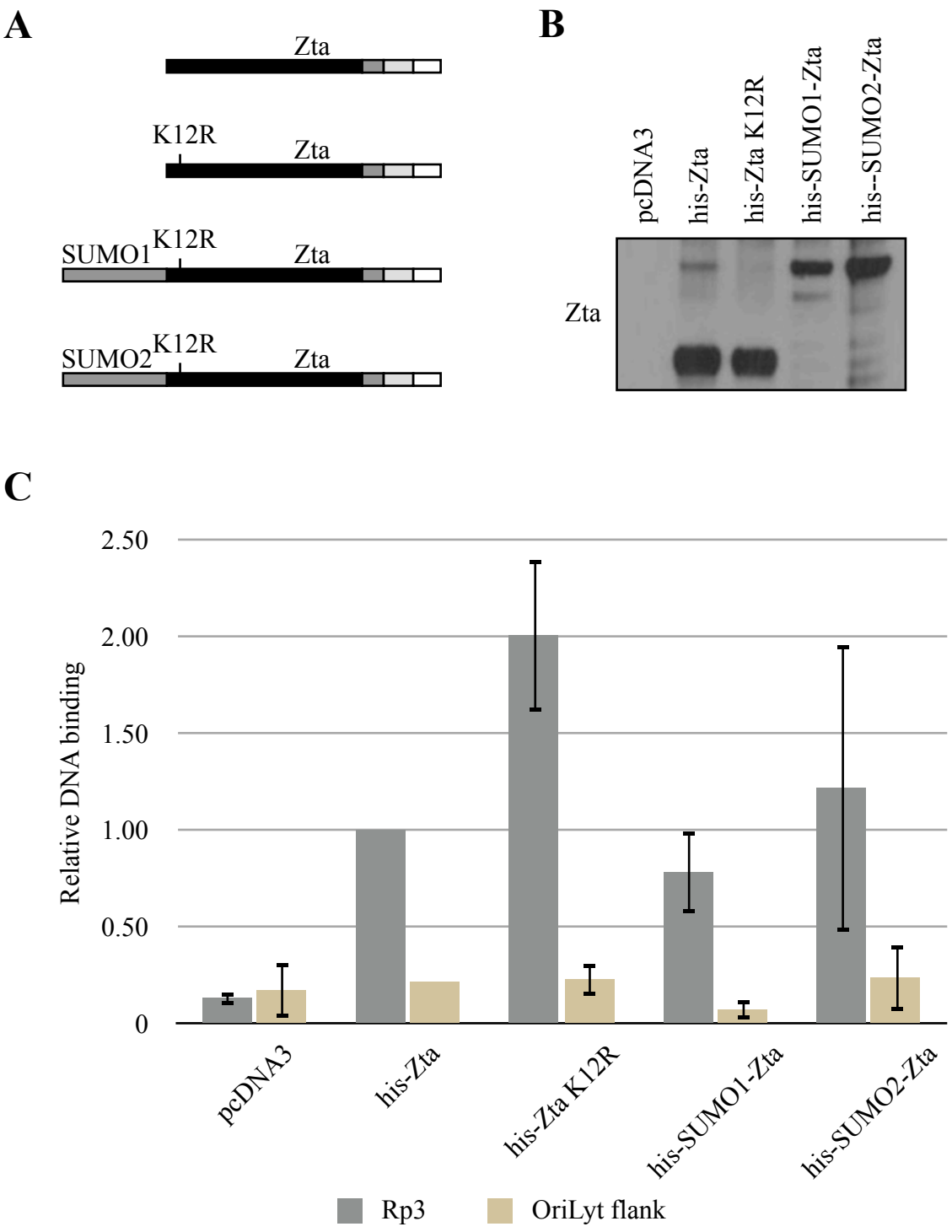


Figure 4.6 SUMO fusion proteins and Lysine 12 mutant can bind DNA *in vivo*. Chromatin immunoprecipitation was performed in HEK 293T EBV-ZKO cells according to the protocol. **A.** Schematic representation of the proteins used. **B.** Western blot analysis using BZ1 antibodies to confirm immunoprecipitation of the proteins with ScZ antibodies. **C.** qPCR analysis using Rp3 primers that overlap Rta promoter in the EBV genome which contains ZREs and has a high affinity for Zta, and Orilyt flank primers, know not to bind Zta. Values were normalised to his-Zta + Rp3. Error bars indicate standard deviation; data from two biological replicates.

4.2.6 SUMO-Zta fusion and Zta K12R proteins can bind DNA *in vivo*.

Having validated the *in vitro* DNA binding of SUMO fusion and K12R, Zta variants, an *in vivo* DNA binding assay was also devised. SUMO1-Zta, his-SUMO2-Zta, his-Zta K12R, his-Zta and the empty pcDNA3 expression plasmids were transfected into the HEK 293T EBV-ZKO cells and processed according to the ChIP protocol. After the immunoprecipitation step with ScZ antibody (that recognises Zta), a portion of the samples were used for western blotting with another Zta binding antibody, BZ1, to confirm expression and precipitation of the desired proteins. Figure 4.6 (B) clearly shows the expression of all the Zta variants including the his-SUMO1-Zta and his-SUMO2-Zta that, as expected, have migrated through the gel the same distance as the band that corresponds to the higher molecular weight form of Zta, which is missing in the his-Zta K12R mutant. The remaining portion of the samples, were fully processed following the ChIP protocol and analysed by quantitative PCR with primers spanning Rp3 (strong Zta binding) and OriLyt flank (background Zta binding) regions of the EBV genome. All of the proteins showed considerable DNA binding to the Rp promoter region, compared to background (OriLyt flank) [Figure 4.6 (C)]. Together with previous results, these findings showed that the SUMO fusion proteins did not alter the DNA binding ability by Zta and could be used as a working model for investigating the effects of SUMOylation on Zta.

4.2.7 SUMO inhibits *CIITA* repression but not *BHLF1* activation by Zta.

Once the binding ability had been established, the fusion proteins were used in a Luciferase assay to study the effects on activation of *BHLF1* and repression of *CIITA*.

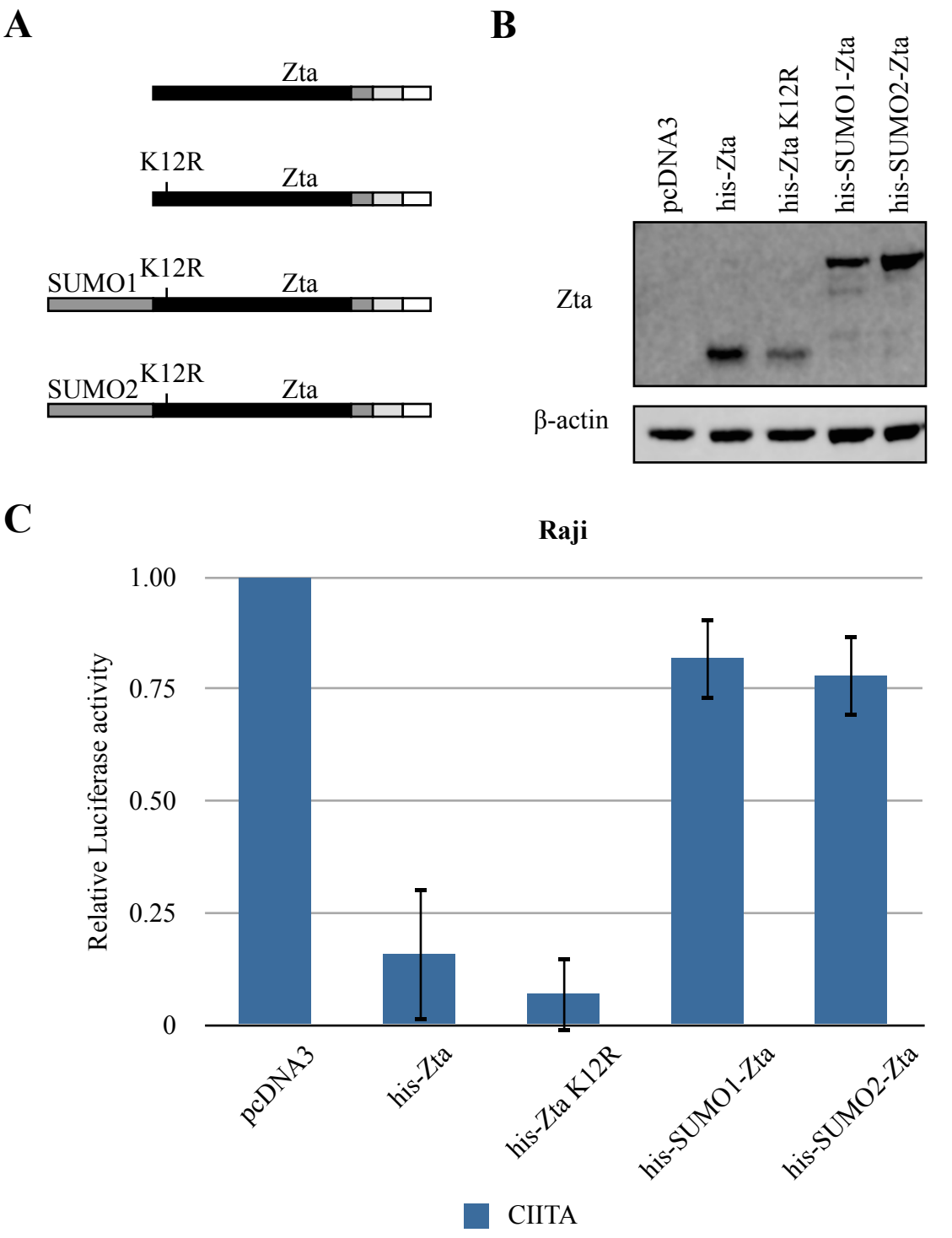


Figure 4.7 SUMOylation of Zta inhibits repression of the CIITA promoter. Raji cells were transfected with the corresponding plasmids and processed according to Luciferase assay protocol. **A.** Schematic representation of the proteins used **B.** Western blot analysis using BZ1 (Zta) and anti-β actin antibodies. **C.** Luciferase assay data showing the effects of various his-Zta protein constructs on the CIITA promoter in Raji cells. The values were adjusted to total protein concentration and normalised to pcDNA3. Error bars indicate standard deviation; data from three biological replicates.

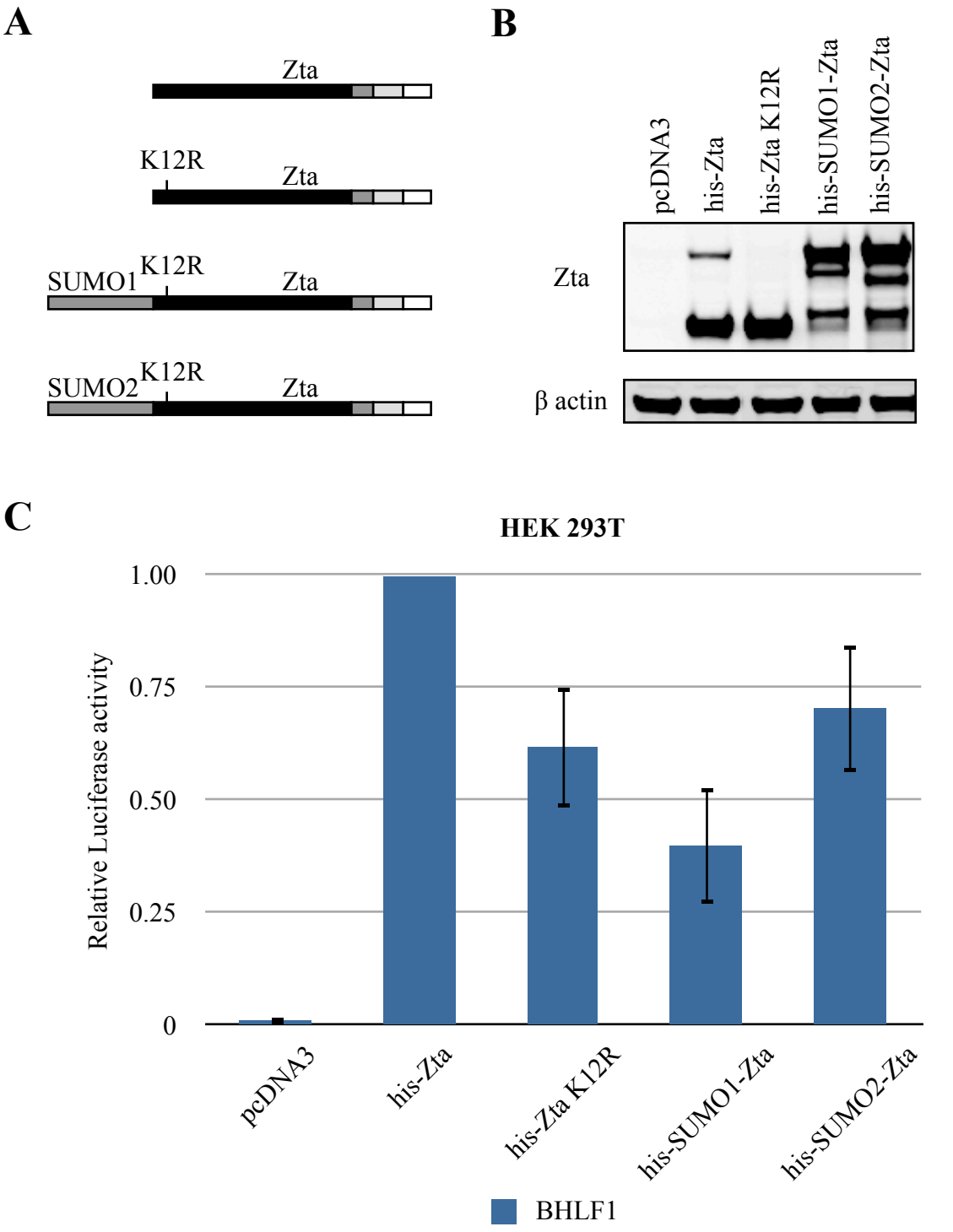


Figure 4.8 SUMOylation of Zta does not inhibit activation of BHLF1.
HEK 293T cells were transfected with the corresponding plasmids and processed according to Luciferase assay protocol. **A.** Schematic representation of the proteins used **B.** Western blot analysis using BZ1 (Zta) and anti-β actin antibodies. **C.** Luciferase assay data showing the effects of various his-Zta protein constructs on the BHLF1 promoter in HEK 293T cells. The values were adjusted to total protein concentration and normalised to his-Zta. Error bars indicate standard deviation; data from three biological replicates.

The data in Figure 4.7 show that the SUMO fusion proteins have been severely impaired in their ability to repress the pIII promoter of *CIITA* in Raji cells, compared to wild type Zta. In addition to that, the mutation of Lysine 12 to arginine did not have an effect on Zta's function, indicating that this residue is not required for repression of the *CIITA* promoter [Figure 4.7 (C)].

Interestingly, his-Zta K12R, his-SUMO1-Zta and his-SUMO2-Zta were all able to activate the *BHLF1* promoter, but at statistically lower levels than his-Zta in HEK 293T cells [Figure 4.8 (C)]. The t-test p-values for his-Zta activation of the *BHLF1* promoter compared to that of his_Zta K12R, his-SUMO1-Zta and his-SUMO2-Zta activation were all lower than 0.01. These findings add to the overall complexity of the processes involved, as previously published data showed that mutation of Lysine 12 increases the activation of *BMRF1* promoter more than ten fold compared to wild type Zta, and that the overexpression of SUMO1 abolishes *BMRF1* and *BRLF1* promoter transactivation by Zta to almost background levels, but does not have an effect on the *BZLF1* promoter in HeLa cells (Adamson, 2005).

Together, the findings in Figures 4.7 and 4.8 point at differences in the molecular mechanisms of promoter activation of *BHLF1* and promoter repression of *CIITA*. The evident dissimilarity prompted further investigations.

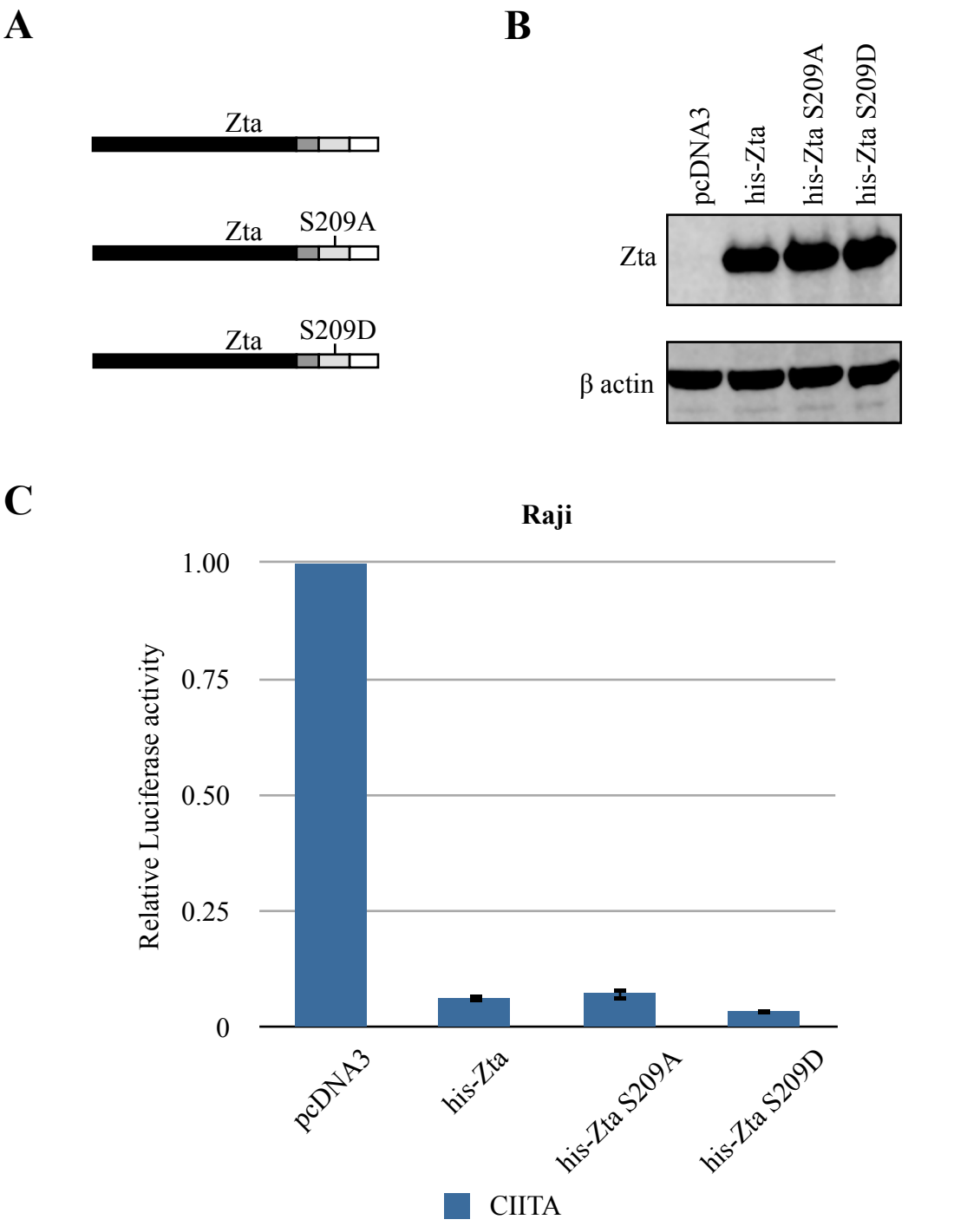


Figure 4.9 Serine 209 phosphorylation of Zta does not have an effect on the repression of the CIITA promoter.
Raji cells were transfected with the corresponding plasmids and processed according to Luciferase assay protocol. **A.** Schematic representation of the proteins used **B.** Western blot analysis using BZ1 (Zta) and anti-β actin antibodies. **C.** Luciferase assay data showing the effects of his-Zta and Serine 209 mutants on the CIITA promoter in Raji cells. The values were adjusted to total protein concentration and normalised to pcDNA3. Error bars indicate standard deviation; data from two biological replicates.

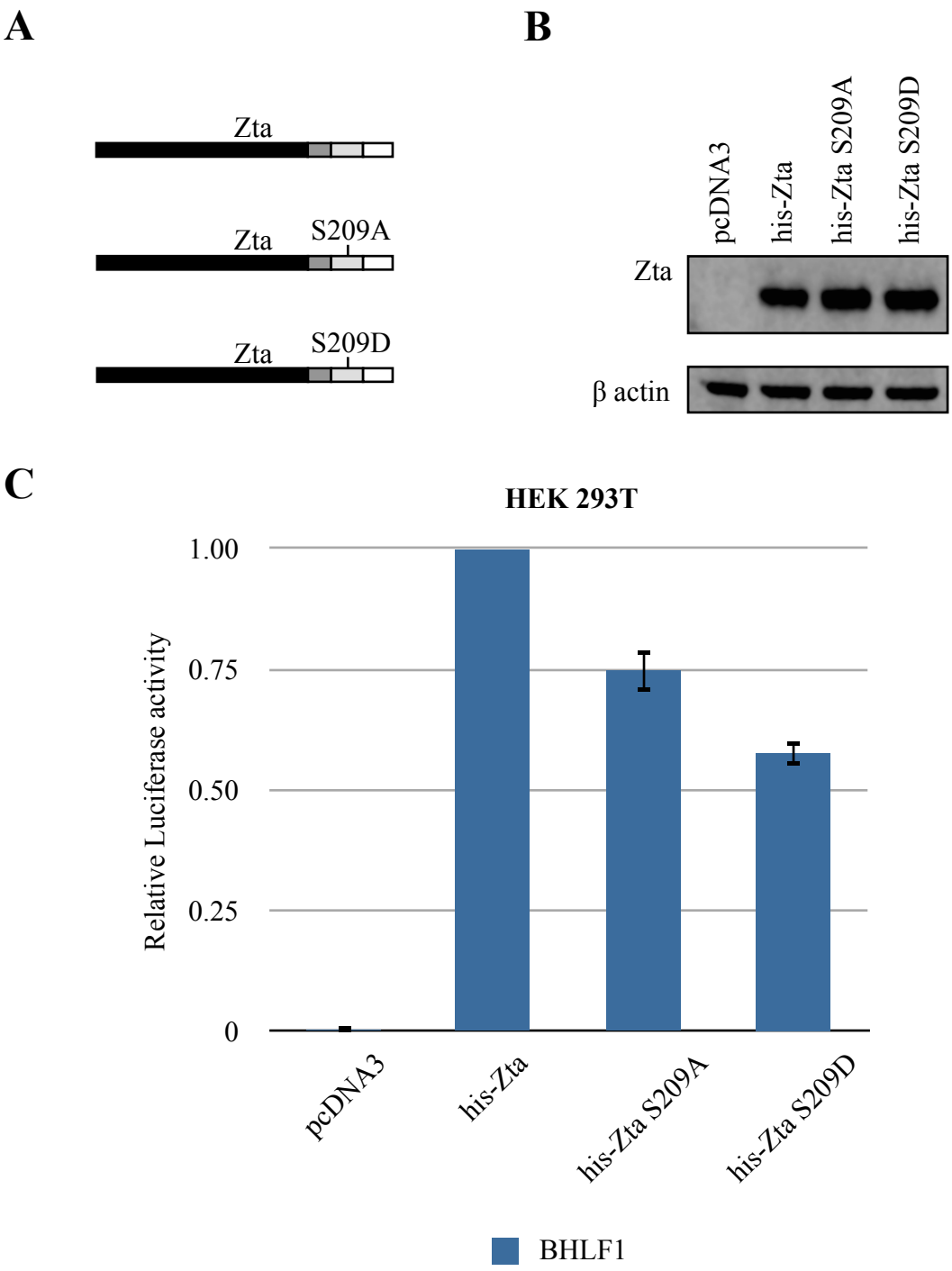


Figure 4.10 Serine 209 phosphorylation of Zta does not have an effect on the activation of the BHLF1 promoter.
HEK 293T cells were transfected with the corresponding plasmids and processed according to Luciferase assay protocol. **A.** Schematic representation of the proteins used **B.** Western blot analysis using BZ1 (Zta) and anti-β actin antibodies. **C.** Luciferase assay data showing the effects of his-Zta and Serine 209 mutants on the BHLF1 promoter in HEK 293T cells. The values were adjusted to total protein concentration and normalised to his-Zta. Error bars indicate standard deviation; data from two biological replicates.

4.2.8 Zta's Serine 209 does not play a role in either activation of *BHLF1* or repression of *CIITA*.

VPK can phosphorylate Zta on Serine 209 and as a result decrease its capacity to transactivate the *BZLF1* promoter in COS-1 cells (Asai et al., 2009). To check if Serine 209 or the phosphorylated version of the residue are important for activation and repression by Zta, the phosphorylation defective his-Zta S209A and the phosphomimic his-Zta S209D, mentioned in the previous chapter, were co-transfected with the model promoters. No significant change in the repression levels of the *CIITA* promoter in Raji cells could be observed by these mutants compared to wild type Zta, all of which had equal levels of protein expression [Figure 4.9]. The data in the Figure 4.10 (C) show that the *BHLF1* promoter was considerably activated above background by all Zta variants in HEK 293T cells [Figure 4.10]. A statistical analysis (t-test) of all the available data showed no significant difference in the activation of the *BHLF1* promoter by his-Zta and his-Zta Serine 209 mutants. These results imply that neither Serine 209, nor the phosphorylation status of this amino acid, are required for Zta to repress the *CIITA* promoter, or activate the *BHLF1* transcription.

4.2.9 The transactivation domain of Zta is crucial for both promoter repression and activation.

The transactivation (TA) domain of Zta plays a central role in the protein's ability to perform various roles, like activating gene expression and initiating DNA replication from OriLyt. The deletion of this part of the protein prevents Zta from performing these important functions, but does not stop direct binding to DNA (Flemington et al., 1991; Sarisky et al., 1996). Since both repression of *CIITA* and activation of *BHLF1* were shown to be dependent on Zta binding to ZREs, a Zta ΔTA mutant was constructed to

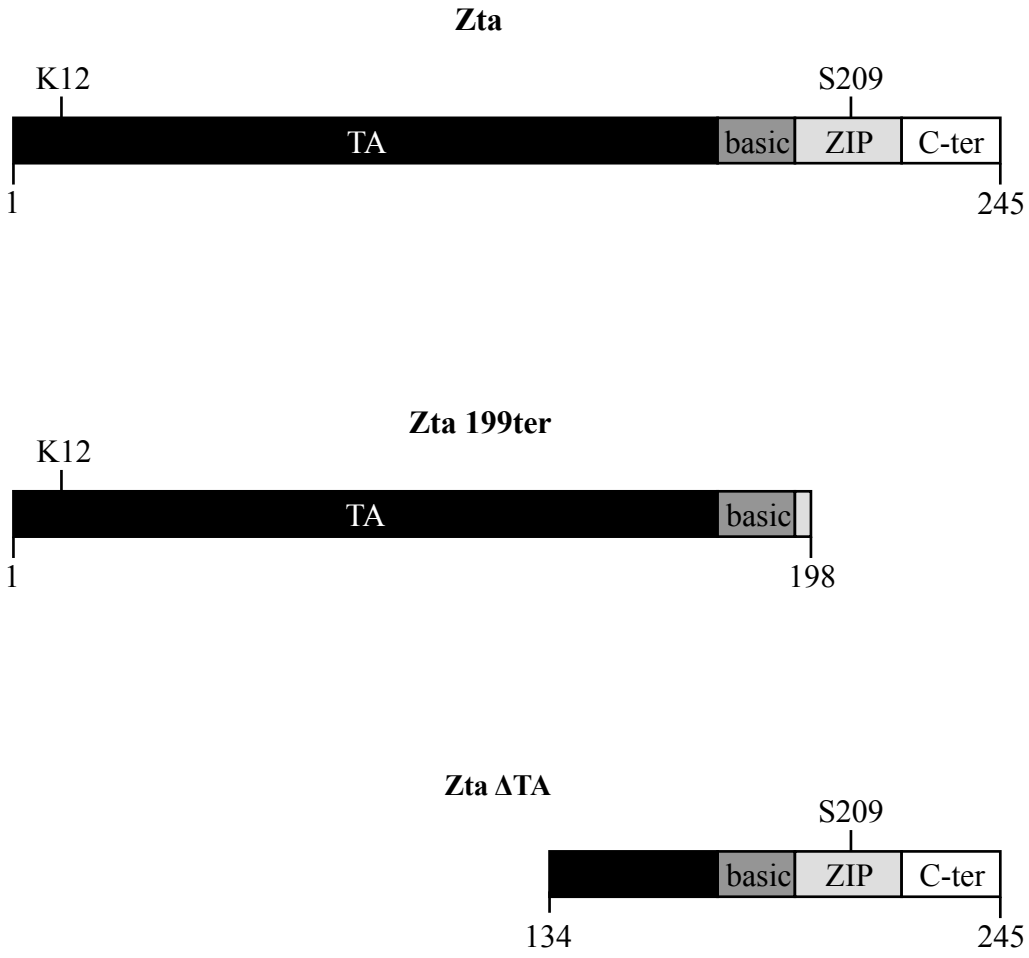


Figure 4.11 Schematic diagram of Zta, Zta 199ter and Zta ΔTA primary structure.
The transativation domain (TA), DNA binding region (basic), leucine zipper domain (ZIP) and the carboxy terminal region (C-ter), as well as the position of Lysine 12 and Serine 209 are shown.

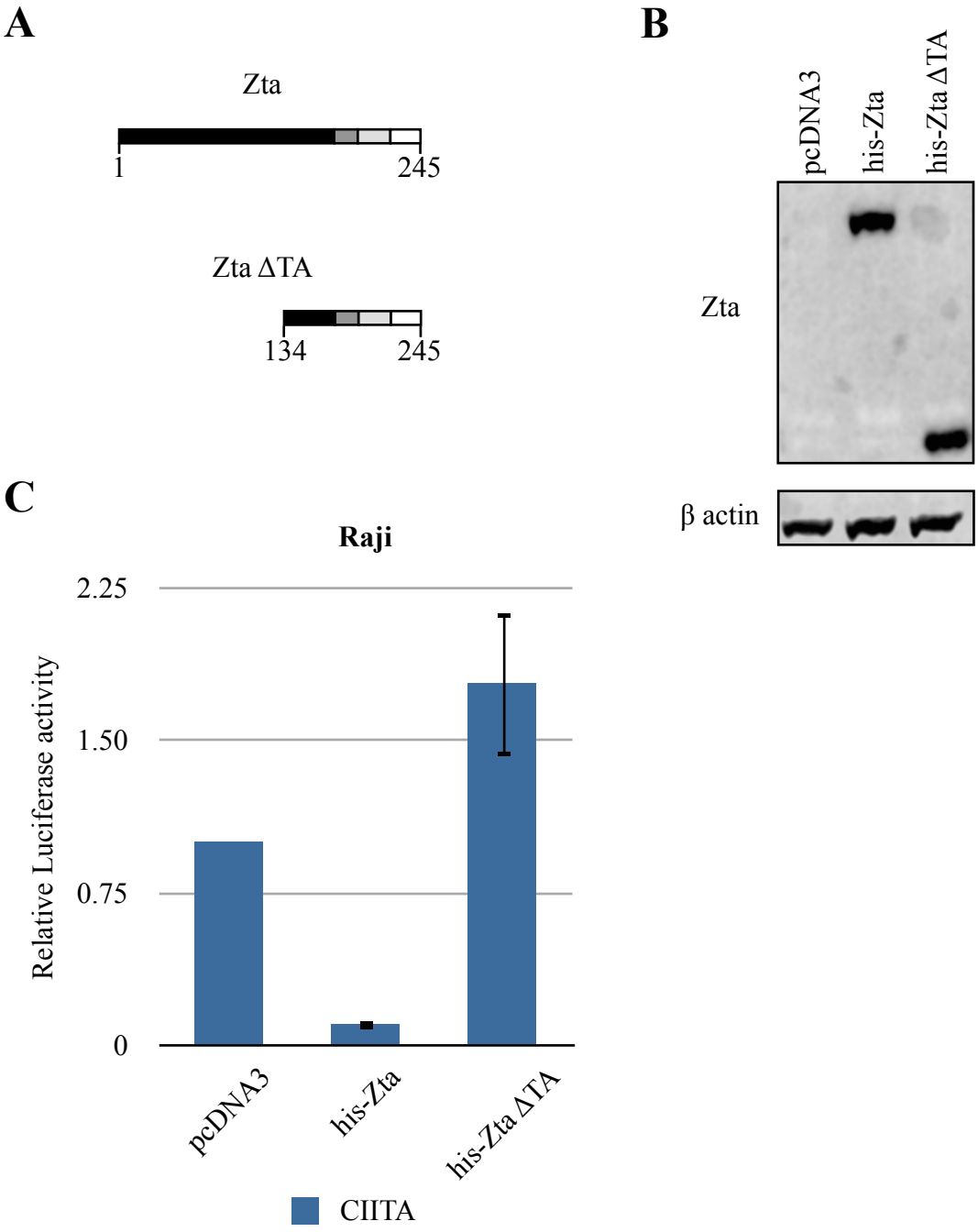


Figure 4.12 Transactivation domain of Zta is essential for the repression of the CIITA promoter.
Raji cells were transfected with the corresponding plasmids and processed according to Luciferase assay protocol. **A.** Schematic representation of the proteins used. **B.** Western blot analysis using BZ1 (Zta) and anti-β actin antibodies. **C.** Luciferase assay data showing the effects of his-Zta and his-Zta ΔTA on the CIITA promoter in Raji cells. The values were adjusted to total protein concentration and normalised to pcDNA3. Error bars indicate standard deviation; data from two biological replicates.

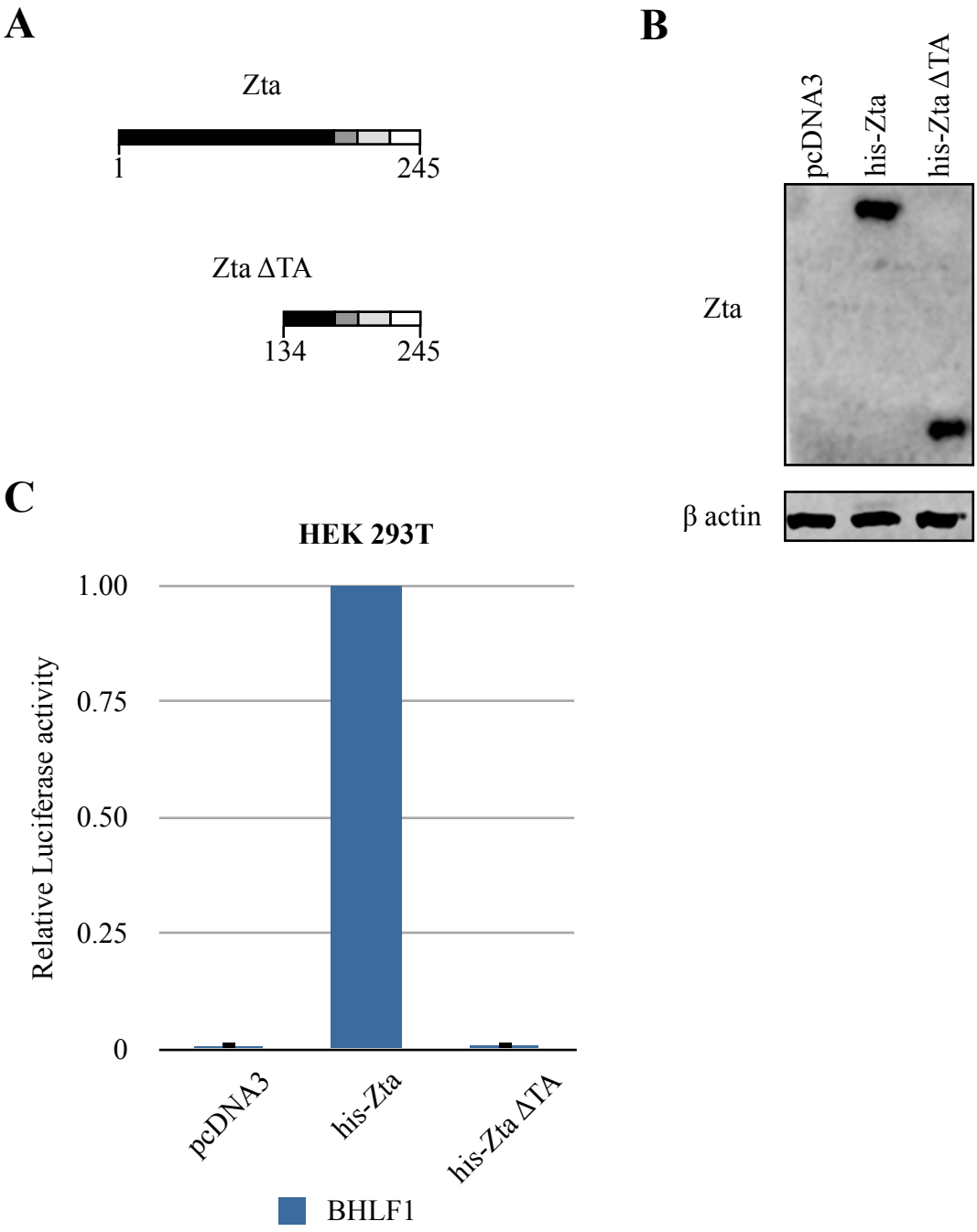


Figure 4.13 Transactivation domain of Zta is essential for the activation of the BHLF1 promoter.
HEK 293T cells were transfected with the corresponding plasmids and processed according to Luciferase assay protocol. **A.** Schematic representation of the proteins used. **B.** Western blot analysis using BZ1 (Zta) and anti-β actin antibodies. **C.** Luciferase assay data showing the effects of his-Zta and his-Zta ΔTA on the BHLF1 promoter in HEK 293T cells. The values were adjusted to total protein concentration and normalised to his-Zta. Error bars indicate standard deviation; data from two biological replicates.

determine if binding to DNA alone is sufficient to elicit an effect in either of the Luciferase assay model systems. The his-Zta ΔTA was designed to have the first 133 N-terminal residues truncated. The DNA sequence coding for the required methionine, followed by 6 histidines placed in front of the remaining 112 C-terminal amino acids of Zta, made up the final ORF [Figure 4.11]. The same procedure undertaken for the SUMO fusion proteins was followed to obtain the desired expression vector (also in pcDNA3).

When his-Zta ΔTA was transiently transfected into Raji and HEK 293T cells, it proved to be stable enough to have the same expression levels as his-Zta [Figures 4.12 (B) and 4.13 (B)]. Moreover, the Luciferase assays showed that the transactivation domain was essential for transcription activity of Zta in either of the used Luciferase model systems. his-Zta ΔTA was completely incapable of repressing *CIITA* promoter in Raji cells [Figure 4.12 (C)] or activating *BHLF1* promoter in HEK 293T cells [Figure 4.13 (C)].

4.2.10 The effects of C-terminal truncations of Zta on transcriptional control.

Having determined of the effects of N-terminal SUMO fusion and N-terminal truncation on Zta's transcptional capacity, it was then decided to investigate the effects of mutation of the C-terminus of Zta. The protein constructs, Zta 199ter and Zta 243ter previously published in Bailey's et al. (2009) work were obtained, together with Zta AAA, which was studied in the laboratory and showed similar properties to Zta 243ter (unpublished data). All three protein mutants needed to be subcloned into a pcDNA3 expression vector and modified to contain the (6x) polyhistidine sequence, to maintain

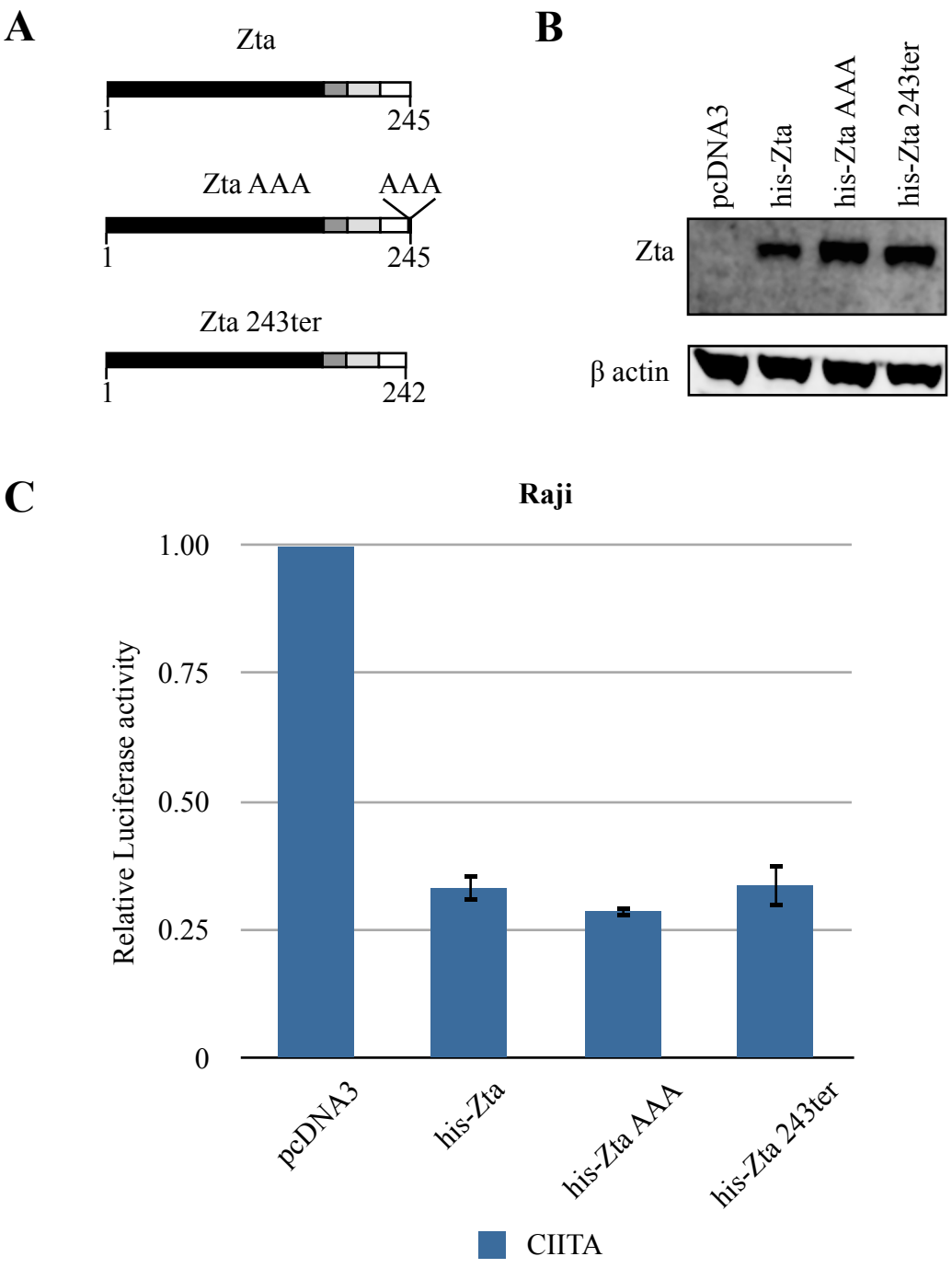


Figure 4.14 The extreme C-terminus of Zta is not required for repression of CIITA.
Raji cells were transfected with the corresponding plasmids and processed according to Luciferase assay protocol. **A.** Schematic representation of the proteins used **B.** Western blot analysis using BZ1 (Zta) and anti-β actin antibodies. **C.** Luciferase assay data showing the effects of his-Zta, his-Zta AAA and his-Zta 243ter on the CIITA promoter in Raji cells. The values were adjusted to total protein concentration and normalised to pcDNA3. Error bars indicate standard deviation; data from two biological replicates.

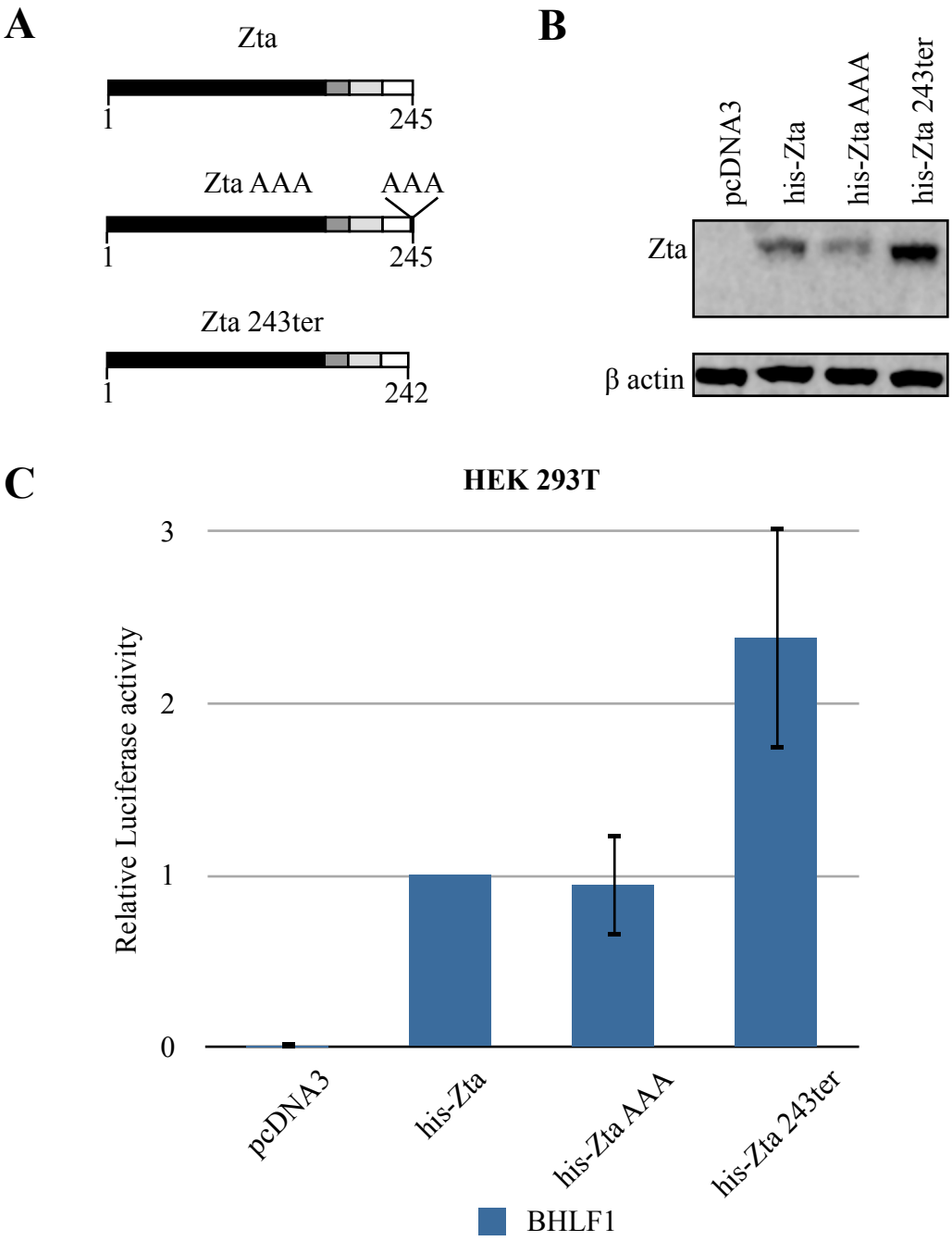


Figure 4.15 The extreme C-terminus of Zta is not required for activation of BHLF1.
HEK 293T cells were transfected with the corresponding plasmids and processed according to Luciferase assay protocol. **A.** Schematic representation of the proteins used. **B.** Western blot analysis using BZ1 (Zta) and anti-β actin antibodies. **C.** Luciferase assay data showing the effects of his-Zta, his-Zta AAA and his-Zta 243ter on the BHLF1 promoter in HEK 293T cells. The values were adjusted to total protein concentration and normalised to his-Zta. Error bars indicate standard deviation; data from three biological replicates.

consistency with the Zta variants used in previous experiments. This was achieved by designing primers that spanned the Zta mutants ORFs, contained the needed restriction enzyme sites (BamH1, EcoRI) and incorporated the his-tag in the forward running primer. The ORF sequences (in the original pBabe backbones) were then put through a PCR reaction, cloned into the pcDNA3 plasmid, sequenced for errors and amplified according to the protocol. Data show [Figures 4.14 and 4.15] that the extreme carboxy terminal mutants, his-Zta AAA and his-Zta 243ter, did not elicit a noticeable effect on repression of the pIII promoter in Raji cells or transactivation of *BHLF1* in HEK 293T cells, compared to wild type Zta. The overactivation of *BHLF1* by his-Zta 243ter could be explained by higher protein level expression seen in Figure 4.15 (B).

The 199ter mutant of Zta has the dimerisation zipper domain truncated, [Figure 4.11] and as such is unable to bind DNA and activate gene expression or initiate lytic replication (Bailey et al., 2009). Thus, as expected, the his-Zta 199ter mutant was incapable of activating the *BHLF1* in HEK 293T cells [Figure 4.16 (C)]. Surprisingly, the *CIITA* promoter in Raji cells was still repressed by the dimerisation and DNA binding incapable his-Zta 199ter [Figure 4.17 (C)]. However, the 199ter mutant could not achieve the same level of downregulation as the unmodified Zta. These findings suggest that apart from the reported DNA binding dependent pathway, an alternative mechanism of the pIII repression by Zta exists.

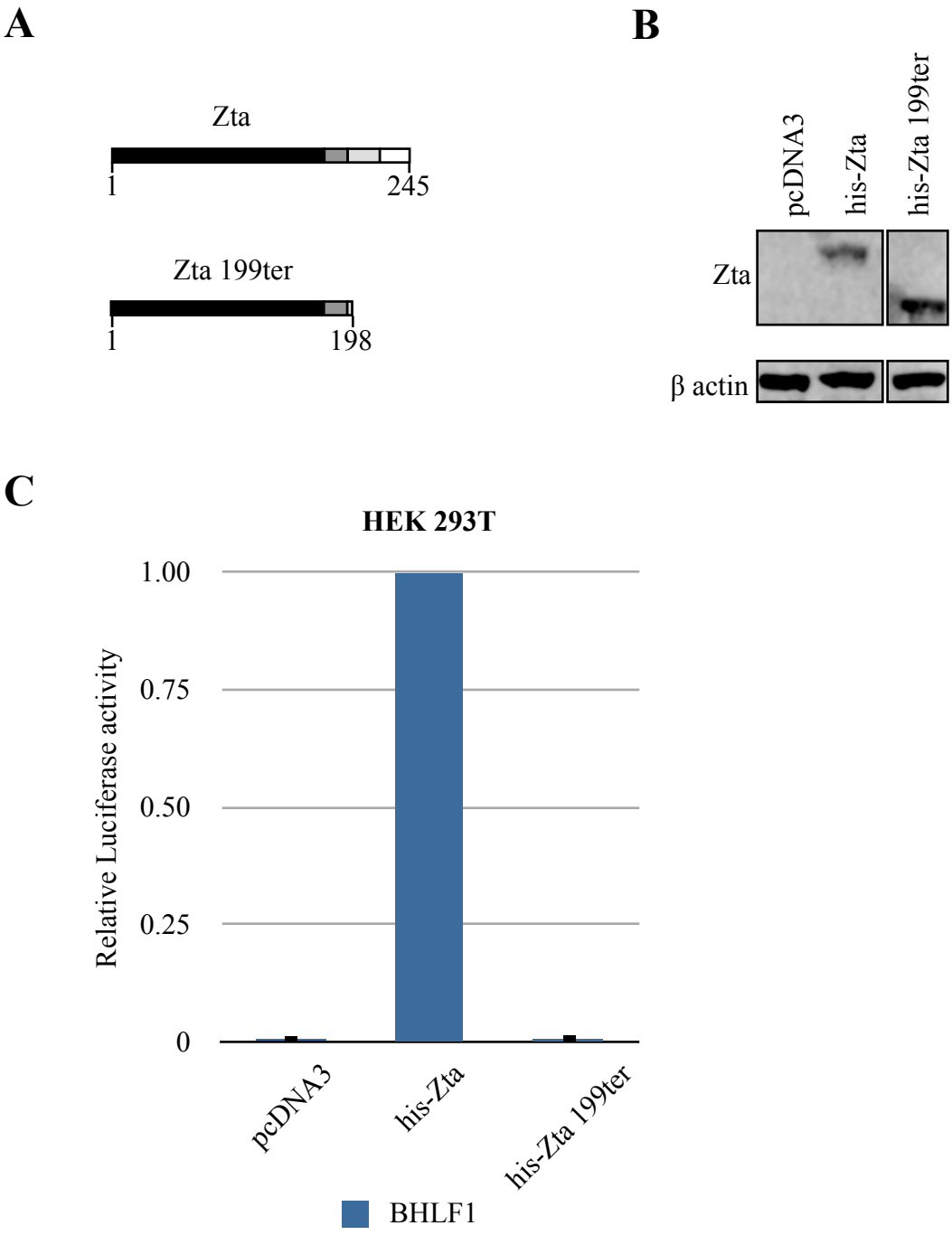


Figure 4.16 Zipper domain of Zta is required for activation of BHLF1.
HEK 293T cells were transfected with the corresponding plasmids and processed according to Luciferase assay protocol. **A.** Schematic representation of the proteins used. **B.** Western blot analysis using ScZ (Zta) and anti-β actin antibodies. **C.** Luciferase assay data showing the effects of his-Zta and his-Zta 199ter on the BHLF1 promoter in HEK 293T cells. The values were adjusted to total protein concentration and normalised to his-Zta. Error bars indicate standard deviation; data from four biological replicates.

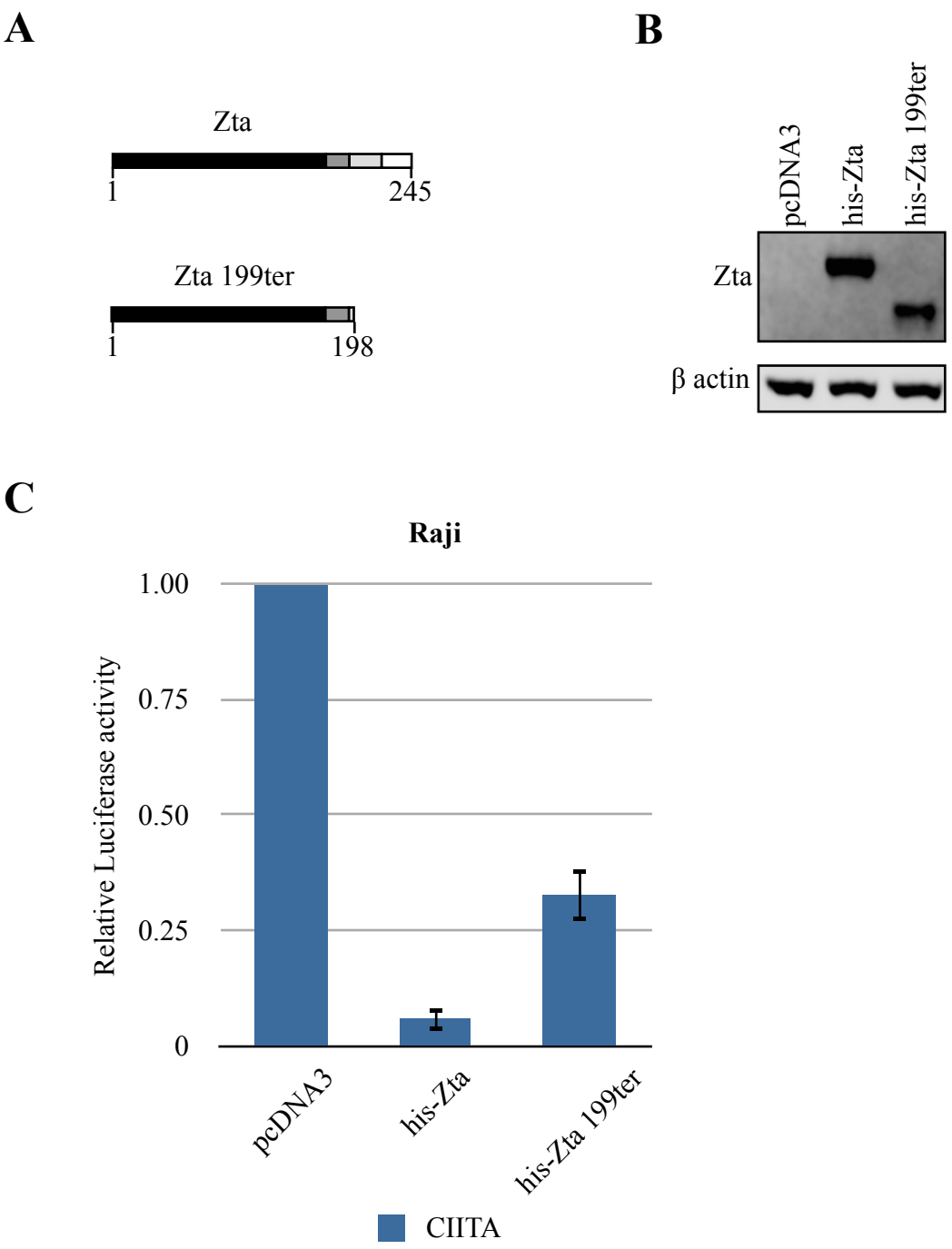


Figure 4.17 Zipper domain of Zta is not required for repression of CIITA. Raji cells were transfected with the corresponding plasmids and processed according to Luciferase assay protocol. **A.** Schematic representation of the proteins used. **B.** Western blot analysis using ScZ (Zta) and anti-β actin antibodies. **C.** Luciferase assay data showing the effects of his-Zta and his-Zta 199ter on the CIITA promoter in Raji cells. The values were adjusted to total protein concentration and normalised to pcDNA3. Error bars indicate standard deviation; data from three biological replicates.

4.2.11 An attempt to determine the exact domains of Zta involved in *CIITA* repression.

To pinpoint the exact domains involved in the *CIITA* DNA binding independent repression, a series of mutants shorter than Zta 199ter were designed. The constructs graphically represented in Figure 4.18 (B) were created by the method described previously in the chapter 4.2.4. Unfortunately, all of the attempts to express these proteins in Raji cells turned out to be unsuccessful [Figure 4.18 (A)]. Various factors could have influenced this, including altered protein stability and turnover due to shorter polypeptide sequence. It is also important to mention that the studied Zta isoforms [Figure 4.18] were designed to have shorter nucleotide sequences than the wild type Zta; unlike Zta 199ter, which was generated by site-directed mutagenesis of the full Zta sequence subcloned in an expression vector (Bailey et al., 2009). Therefore, the mRNA stability of these shorter constructs could have been affected in Raji cells. A control for future experiments could include a Zta 199ter also designed with an abrogated sequence, rather than with a stop codon in a full Zta sequence.

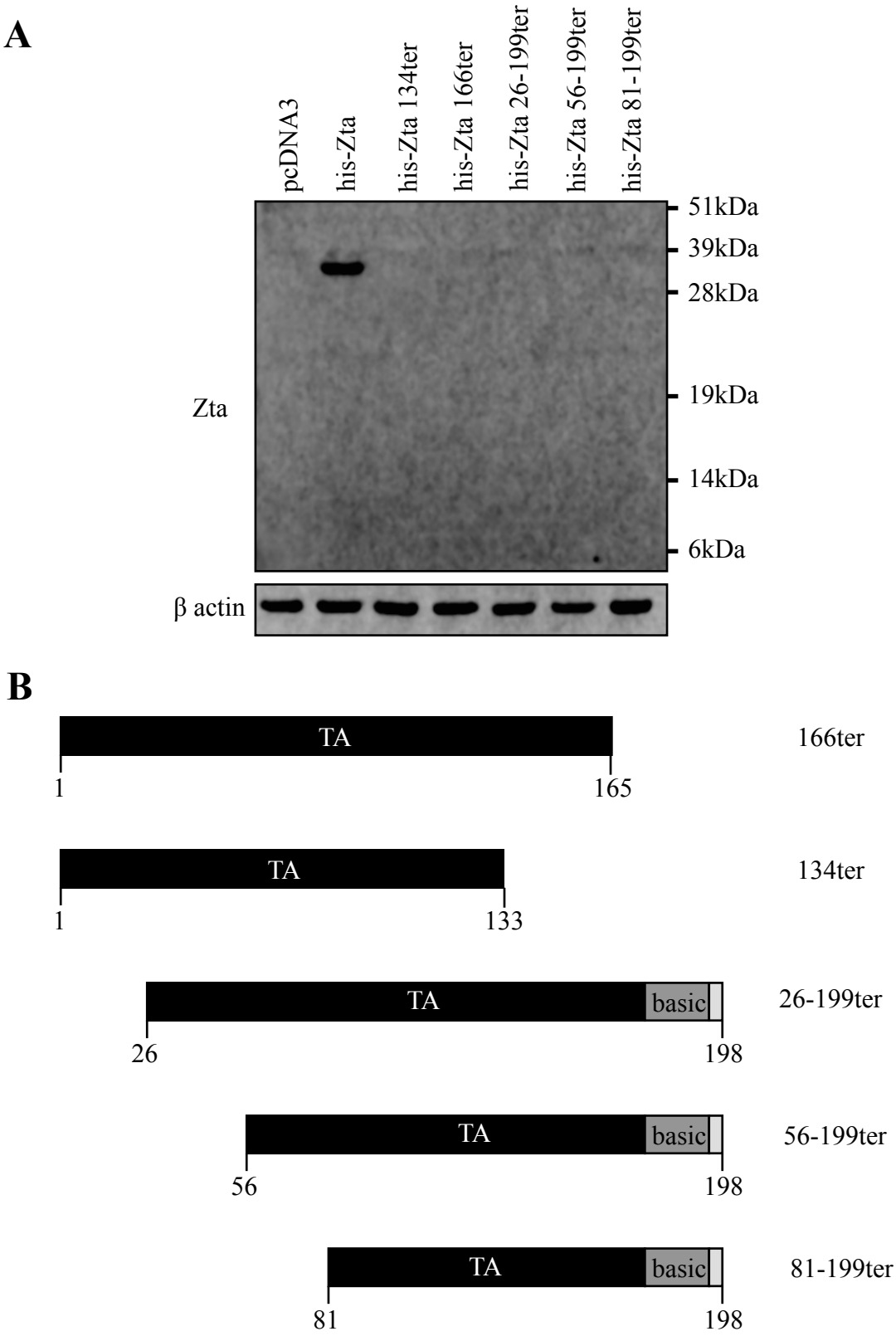


Figure 4.18 Inability to express short Zta mutants.
A. Raji cells were transfected with the corresponding plasmids, harvested 48h later in 8M Urea and analysed using anti-polyhistidine (for Zta) and anti-β actin antibodies.
B. Graphical representation of the primary structure of the short Zta mutants designed

4.3 Chapter discussion.

The focus of this chapter was to investigate the differences in Zta's ability to activate the viral *BHLF1* promoter and repress the cellular *CIITA* promoter, the results of which are summarised in Figure 4.19. *BHLF1* gene product is one of the most common mRNA species expressed during the lytic cycle. It is thought that this mRNA forms an R-loop with the NotI repeat region within the *BHLF1* gene, allowing BALF2 protein to bind to the oriLyt and help initiate viral lytic replication (Rennekamp and Lieberman, 2011). Additionally, the *BHLF1* promoter forms part of the oriLyt, and is crucial for recruiting proteins including Zta, that are essential for efficient DNA replication during the lytic cycle (Schepers et al., 1996). CIITA is a cellular transcription factor that is indispensable for the expression of the class II Major Histocompatibility Complex and the subsequent antigen presentation mediated through this pathway (Steimle et al., 1994). The *CIITA* gene has a complex promoter region, with pIII being the principle site of transcription initiation in B cells (Muhlethaler-Mottet et al., 1997). Both activation of the *BHLF1* promoter, and repression of pIII of *CIITA* by Zta, was shown to be dependent on direct binding to the ZREs found within these promoters (Li et al., 2009; Schepers et al., 1996).

Zta has a complex protein structure that can be roughly subdivided into the amine terminal transactivation domain and the carboxy terminal region that contains the bZIP required for dimerisation and DNA contact. Transcriptional activation by Zta is reliant on both the bZIP region as well as the TA domain. It is thought that recruitment of such factors as TFIIA-TFIID to the DNA through the TA domain, help Zta initiate gene transcription (Ellwood et al., 1999). Direct transcriptional repression through DNA binding

on the other hand, has not been studied in great detail. In addition to a multifunction protein structure, Zta's role can be fine tuned by post-translational modifications like SUMOylation or VPK mediated phosphorylation of Serine 209 (Asai et al., 2009; Hagemeier et al., 2010).

To investigate if the repression of *CIITA* or the activation of *BHLF1* was dependent on other viral co-factors, AK31 cells were used. AK31 are an EBV negative subclone of the Akata cell line that provides a suitable B cell model system, free from viral infection. The data showed that Zta does not require the other EBV genes to either activate *BHLF1* or repress *CIITA*. These results indicate that Zta alone is involved in the immune evasion, through modulation of the *CIITA* expression.

As SUMOylation can be an important regulator of Zta's transcriptional function, two SUMO-Zta fusion proteins were designed to mimic constitutive SUMOylation of Zta. Together with the S209A, S209D and K12R mutants described in the previous chapter, these protein constructs were used to study the effects of Zta PTMs on the repression of *CIITA* and activation of *BHLF1* promoters. Interestingly, the Lysine 12 mutant did not exhibit a superactivation of *BHLF1*, which was reported for such promoters as Zp, *BALF2* (Murata et al., 2010) and *BMRF1* (Adamson, 2005). The data does however agree with the Deng et al. (2001) publication in which a K12A/F13A Zta could activate *BHLF1* promoter less than 50% compared to the wild type protein. Lysine 12 might be involved in the folding of the TA domain required for promoter activation of *BHLF1* but not other lytic genes. Murata et al. (2010) showed that a SUMO-Zta fusion protein could not activate the Zp promoter above background levels and was unable to

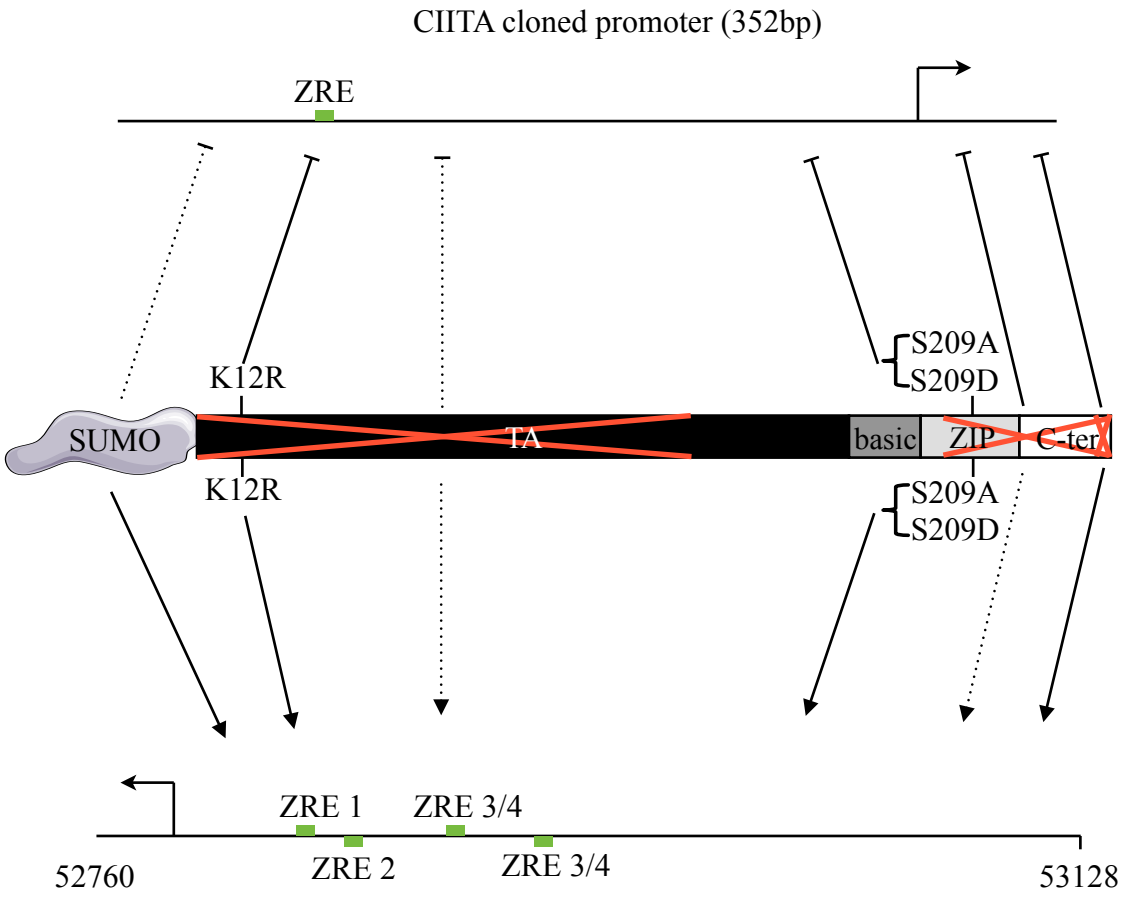
induce the expression of BALF2 and BMRF1 proteins. The results presented in this chapter, on the other hand, revealed that SUMO-Zta chimeric proteins exhibited a decreased activation compared to wild type Zta, but were able to activate the *BHLF1* promoter considerably above background. This suggests that SUMOylation could inhibit Zta's transactivation to various degrees, depending on the promoter region involved. It is however important to remember that the SUMO-Zta chimeras also had the Lysine 12 residue mutated, which might have accounted for the decrease in Zta's transactivation, with SUMO having just a marginal effect. Another indication that the effect of Zta PTMs on *BHLF1* expression is divergent from that on other viral promoters comes from the Serine 209 mutants. Both S209A and S209D Zta variants activated the *BHLF1* promoter similar to the wild type protein, while the results in the previous chapter indicated that the same mutants were associated with an increase in Rta protein levels. The similarity in the effects of S209A and S209D could be explained by the S209D mutation not being able to exhibit a true phosphomimic function and rather acting as a phosphorylation blocking amino acid. Altogether, these results suggest that Zta transactivation of *BHLF1* is less susceptible to the inhibition by SUMOylation and VPK mediated phosphorylation than other lytic promoters. Since both the *BHLF1* promoter and *BHLF1* mRNA are directly involved viral lytic replication, SUMOylation might be a way in which EBV adjusts Zta's function in the later stages of the lytic cycle: from transcription of viral protein-coding genes, towards replication of the EBV genome.

The influence of Zta PTMs on the repression of pIII was also investigated. Neither the Lysine 12 nor the Serine 209 residues were required or involved in this process. SUMO fusion proteins on the other hand, did have a considerable effect on Zta's poten-

tial to repress *CIITA*. It is plausible that the fusion of SUMO could have affected the DNA binding affinity of Zta for the *CIITA* promoter, however this is unlikely, since SUMO-Zta was able to bind DNA both *in vivo* and *in vitro*. Therefore, the DNA binding dependent repression of the *CIITA* promoter is not likely to be due to steric hindrance of the Zta protein in the pIII region and might involve recruitment of other co-factors to the DNA. This theory is supported by the fact that the ΔTA Zta mutant also lost the ability to repress the *CIITA* promoter, indicating that DNA binding alone is not sufficient for the repression.

The most striking results however, came from investigating the carboxy terminus of Zta. While the effects of the C-terminus mutants on the activation of *BHLF1* promoter exhibited a response concurrent with published data, the impact of the truncated proteins on the repression of *CIITA* showed some unexpected results. Most importantly, the dimerisation and DNA binding incapable Zta 199ter mutant (Schelcher et al., 2007), was still able to inhibit the expression from pIII. This suggests that in parallel with the published DNA binding dependent repression (Li et al., 2009), an alternate DNA binding independent mechanism of *CIITA* downregulation by Zta exists. Furthermore, this alternative route of action must be facilitated by a Zta monomer. Unfortunately, the efforts to pin point the exact Zta domains involved have remained elusive.

To investigate the *CIITA* repression further, a pIII promoter mutagenesis approach was employed and described in the subsequent chapter.



Zta modification	Repression	Activation
SUMO fusion to the N-terminus	✗	✔
Lysine 12 mutation	✔	✔
Truncation of the transactivation domain	✗	✗
Serine 209 mutations	✔	✔
Zta 243ter and AAA mutants	✔	✔
Zta 199ter mutant	✔	✗

Figure 4.19 Schematic diagram of the effects of various modifications and truncations of Zta on its function as a suppressor or activator.
The effects of SUMO-fusion, K12R, S209A and S209D mutations, C-ter and N-ter truncations on Zta's ability to repress the CIITA promoter or activate the BHLF1 promoter are shown.

5. Analysis of CIITA's promoter sequence involved in repression by Zta.

5.1 Introduction.

The necessity to regulate the *CIITA* gene stems directly from the need to control the expression of MHC class II molecules in a cell type specific and temporal manner. This issue is partially resolved by differential expression of various *CIITA* promoters (pI, pIII and pIV) in different cell types (Muhlethaler-Mottet et al., 1997). In B cells, transcription initiates mainly from pIII of *CIITA*. However, other cell types (e.g. T cells) also rely on pIII for their *CIITA* expression (Holling et al., 2002; Muhlethaler-Mottet et al., 1997). In addition to that, B cells themselves require the ability to silence the pIII promoter, in order to switch off the MHC class II presentation upon achieving terminal differentiation into plasma cells (Silacci et al., 1994). Therefore pIII of *CIITA* by its nature is a highly regulated and complex DNA region.

Epigenetic mechanisms like chromatin remodelling play an important role in the regulation of pIII. Hypermethylation of this promoter region in murine lymphoma cells is associated with inhibition of CIITA (Murphy et al., 2002). Indeed, chromatin marks linked with the repressed transcription state, like Lysine 9 dimethylation of histone H3, are low in relation to pIII from B cells compared to higher levels observed in terminally differentiated plasma cells. Moreover, 'open' chromatin markers like histone H3 Lysine 9 and Lysine 27 acetylation are lost in plasma cells (Green et al., 2006). This effect, at least in part, might be mediated through Blimp-1 protein binding to pIII of *CIITA* (Piskurich et al., 2000) which is able to recruit histone deacetylases to the DNA, that

remove acetyl groups from Lysine residues, ultimately inhibiting gene expression (Yu et al., 2000).

The importance of the pIII promoter is also highlighted by low levels of small nucleotide polymorphisms (SNPs), indicating potential high sequence conservation (Castle, 2011; Janitz et al., 2001; Patarroyo et al., 2002; Zhao et al., 2005). A number of DNA elements in this promoter region have been confirmed as binding sites for cellular proteins. These include two activation response elements, ARE-1 and ARE-2 critical for efficient transcription and five non-essential sequences: sites A, B and C, as well as two E-box motifs. Moreover, three cAMP responsive elements (CREs) located downstream and one immediately upstream of the TSS were also found to be important for promoter activity (Ghosh et al., 1999; van der Stoep et al., 2004; van der Stoep et al., 2002). ARE-2 also incorporates a CRE motif in its sequence and as such can bind the closely related cAMP response element-binding protein (CREB) and ATF-1 transcription factors, and subsequently recruit CBP as well. Even though CREB is a ubiquitously expressed protein, the 5' UTR CREs of pIII are involved in the CIITA promoter activation only in B-cells (Raji) and not in T lymphocytes (Jurkat) (van der Stoep et al., 2002). Furthermore, ARE-1, ARE-2 and site C, all need to be free of protein for efficient promoter silencing in plasma cells, which is possibly achieved through earlier discussed chromatin remodelling. It was also demonstrated that ARE-1, site A and site B have an affinity for TEF-2, NF-1 and OTF-1 proteins respectively (Ghosh et al., 1999). Site C and the E-box motifs on the other hand, have the sequence Ets/interferon stimulated response element (ISRE)-consensus element (EICE) and E47 homology site respectively. These sequences are critical in recruiting a complex of IRF-4, PU.1 and E47 proteins

which together, synergistically transactivate the pIII in B lymphocytes but not in plasma or T cells (van der Stoep et al., 2004).

Finally, chromatin conformation capture techniques revealed that a so called HSS1 (hypersensitive site 1) located about 11kb upstream of pIII can bind PU.1 and subsequently pIII, forming a loop structure required for CIITA expression in B cells. The HSS1, similar to pIII, also has elevated euchromatic markers in B cells compared to low levels found in plasma cells (Yoon and Boss, 2010).

Together, the accumulated evidence suggest that temporal control of pIII is achieved through the chromatin remodelling mechanisms. Whereas the cell type specific activity of the pIII promoter is governed by the expression of different combinations of transcription factors. Interestingly, Zta was reported to directly interact with several proteins involved in pIII promoter activation in B cells, like CREB and CBP (Adamson and Kenney, 1999). It is possible that Zta might act through protein-protein interactions to repress the pIII of *CIITA*.

The object of this chapter is to identify DNA sequences involved in the process of pIII repression by Zta, employing a mutagenic approach of the promoter region.

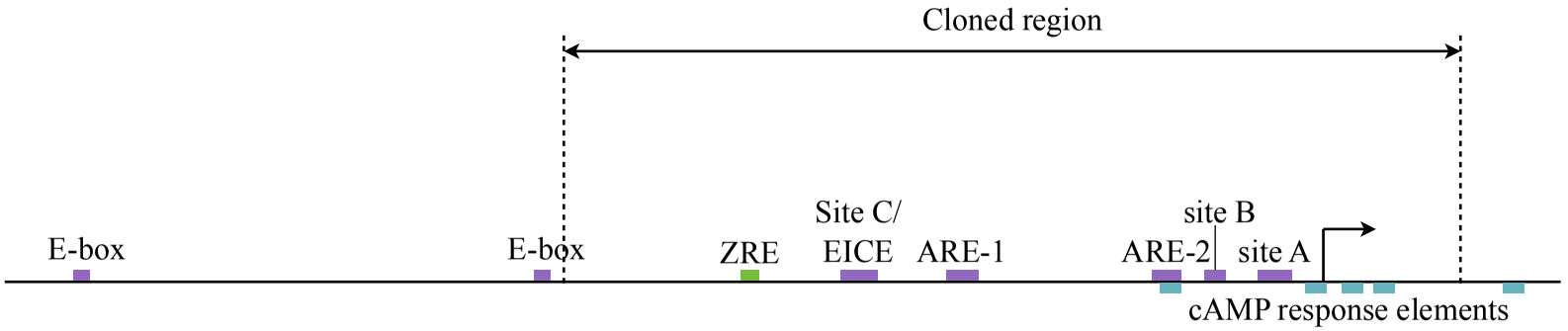


Figure 5.1 Schematic representation of the CIITA pIII promoter.
The published features are shown. E-box motif, EICE (Site C), ARE-1, ARE-2, Site B and Site A are shown in purple. The CREs are shown in blue. Promoter proximal ZRE is shown in green. Dotted lines indicate the cloned *CIITA* region. (Ghosh et al., 1999; Li et al., 2009; van der Stoep et al., 2004; van der Stoep et al., 2002)

5.2 Results.

5.2.1 CIITA ZRE mutations.

As shown in the previous chapter, his-Zta 199ter, which is unable to bind DNA, could also repress pIII of *CIITA*. This surprising discovery pointed at an alternative mechanism of *CIITA* repression by Zta. In order to confirm these results at the DNA level, a promoter mutagenesis approach was employed. First, the ZRE (TGTGCAA) found within the cloned promoter region was substituted for several other sequences. A CIITA null ZRE mutant with the sequence of 'CCCCCTT' was designed to be unrecognisable by Zta and thus unable to form a DNA-protein complex [Figure 5.2 (A)]. Moreover, to assess a potential reversal of repression, the ZRE 3/4 of the BHLF1 promoter, with the sequence of 'TGACACA' was exchanged for the original ZRE in the pIII [Figure 5.2 (A)]. The constructs were ordered, subcloned into pGL3 enhancer plasmid, sequenced for errors, amplified and used in a Luciferase assay. The data show [Figure 5.2 (B)] that his-Zta was able to repress both CIITA null ZRE and CIITA ZRE 3/4 mutant promoters in Raji cells. This confirms that there is a second mechanism of *CIITA* down-regulation by Zta, completely independent of a ZRE sequence and therefore, direct DNA binding.

5.2.2 CIITA promoter truncations.

To establish if the promoter region downstream of the ZRE was important for repression of pIII by Zta, several truncations of the originally cloned CIITA promoter were designed. Compared to the model promoter (CIITA -288+52) all of the four truncation mutants were shorter at the 5' end (downstream of the TSS) terminating either immediately before the ZRE sequence (CIITA -214+52 and CIITA -214+44) or 15bp

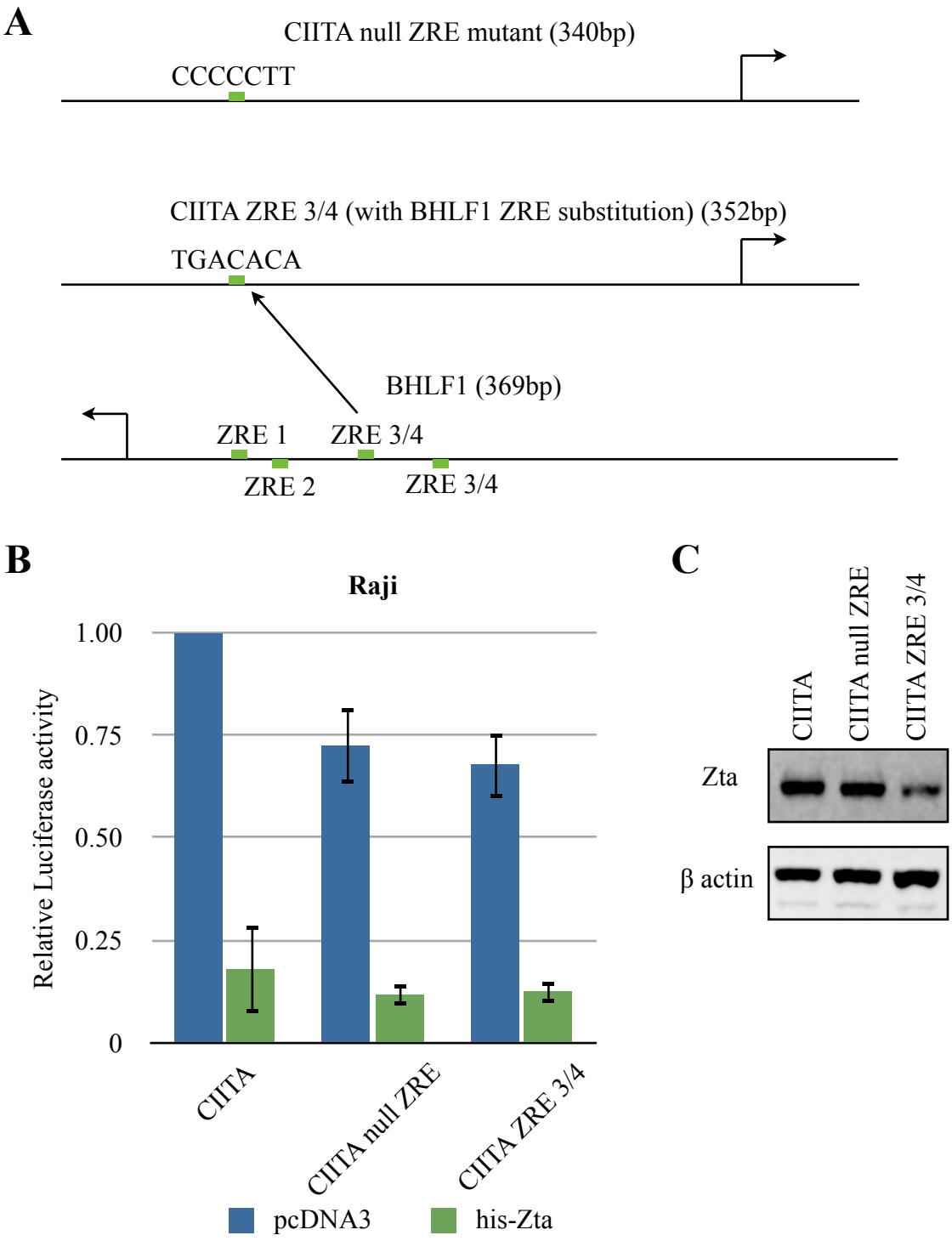


Figure 5.2 Repression of CIITA null ZRE and CIITA ZRE substitution mutants. Raji cells were transfected with his-Zta or empty vector (pcDNA3) and the CIITA pIII, CIITA null ZRE, CIITA ZRE 3/4 and processed according to Luciferase assay protocol. **A.** Schematic representation of the mutants used. **B.** Luciferase assay data showing the effects of his-Zta on the ZRE mutant CIITA promoters in Raji cells. The values were adjusted to total protein concentration and normalised to pcDNA3+ CIITA. Error bars indicate standard deviation; data from two biological replicates. **C.** Western blot analysis of Zta levels using BZ1 (Zta) and anti-β actin antibodies.

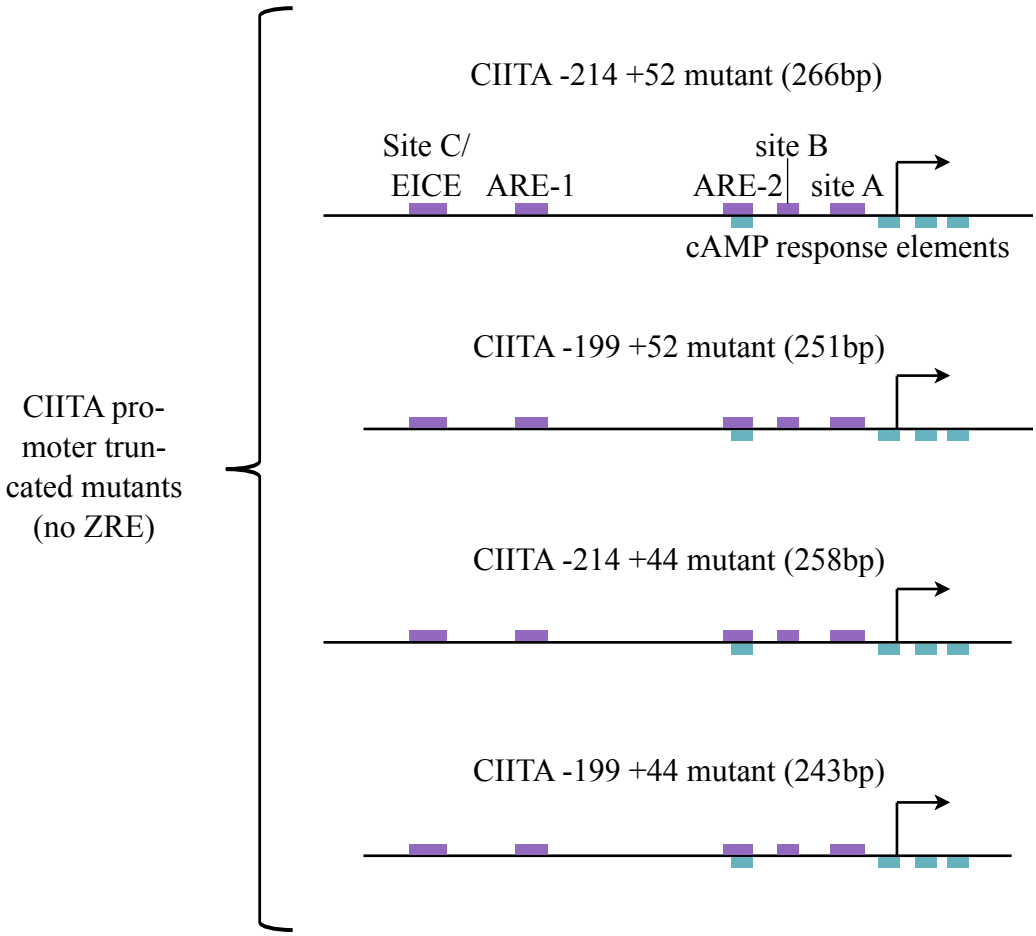


Figure 5.3 CIITA promoter truncated mutants.
Schematic representation of the four CIITA truncation mutants designed. All of the mutants are missing the ZRE and the CIITA -199+44 corresponds to the one published by Li et al.

after (CIITA -199+52 and CIITA -199+44). Two mutants, CIITA -214+44 and CIITA -199+44, were also shorter at the 3' end, (upstream of the TSS), so that the CIITA -199+44 corresponded to the one published by Li et al. (2009) [Figure 5.3]. The constructs were ordered and processed as described earlier. As the data in Figure 5.4 show, all of the mutants were repressed by his-Zta in Raji cells, agreeing with the results mentioned earlier, and indicating that the promoter region downstream of the ZRE is not required for repression. Moreover, the two -199 *CIITA* promoter mutants exhibited a decreased basal activity, indicating that an important, previously unreported DNA element could be situated within that region.

5.2.3 Comparative analysis of promoters repressed by Zta.

Bergbauer et al. (2010) reported that at least 3 EBV promoters are repressed by Zta: *BSLF1, BBLF2/3* and *BSLF2/BMLF1*. Since repression could be dependent on a specific nucleotide sequence e.g. mediated through other DNA binding proteins interacting with Zta, all four promoters (including *CIITA*) were screened for common DNA sequences. PromoterWise (http://www.ebi.ac.uk/tools/psa/promoterwise) web tool was used to find common motifs. However, since the program's algorithm didn't allow for more than 2 promoter comparisons at a time and incorporated a degree of redundancy in the sequence match, after the initial analysis, the data was collated and refined. Only exact sequence matches found between *CIITA* and 2 other mentioned EBV promoters that were at least 7bp long were picked out. From this, 3 sequences in the *CIITA* promoter were identified: 'TGAAGGT', 'TGGGGCCA' shared with *BSLF1* and *BSLF2/BMLF1* and 'AGCTTGGC' shared with *BSLF1* and *BBLF2/3* promoters [Figure 5.5 (A)]. These sequences, together with the ZRE, were then mutated, and the resulting

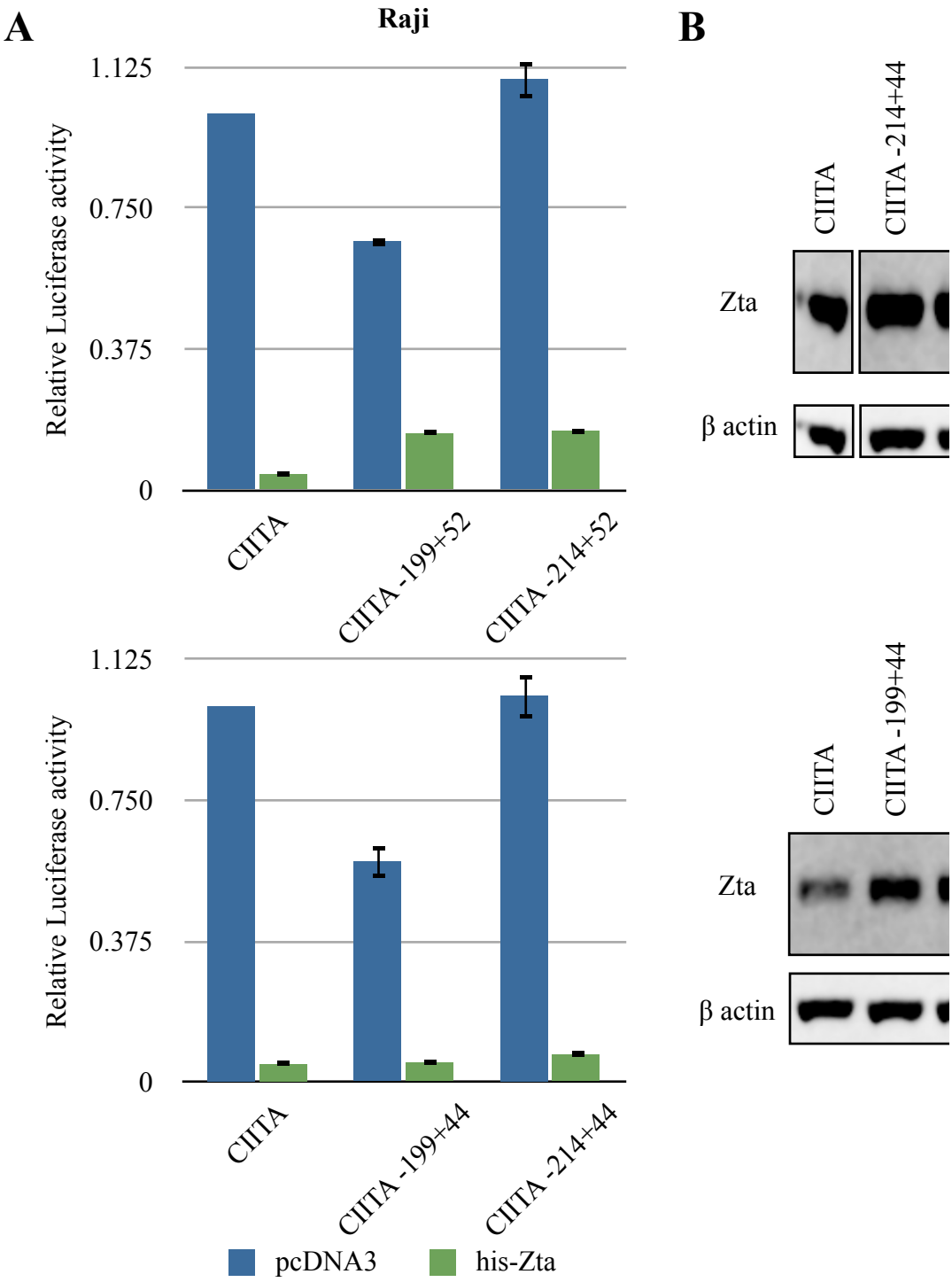


Figure 5.4 Repression of the CIITA truncated mutants.
Raji cells were transfected with his-Zta or empty vector (pcDNA3) and the CIITA -199+52, -214+52, -199+44 or -214+44 and processed according to Luciferase assay protocol. **A.** Luciferase assay data showing the effects of his-Zta on the CIITA promoters in Raji cells. The values were adjusted to total protein concentration and normalised to pcDNA3+CIITA. Error bars indicate standard deviation; data from three technical replicates. **B.** Western blot analysis of the transfected Zta protein levels using BZ1 (Zta) and anti-β actin antibodies.

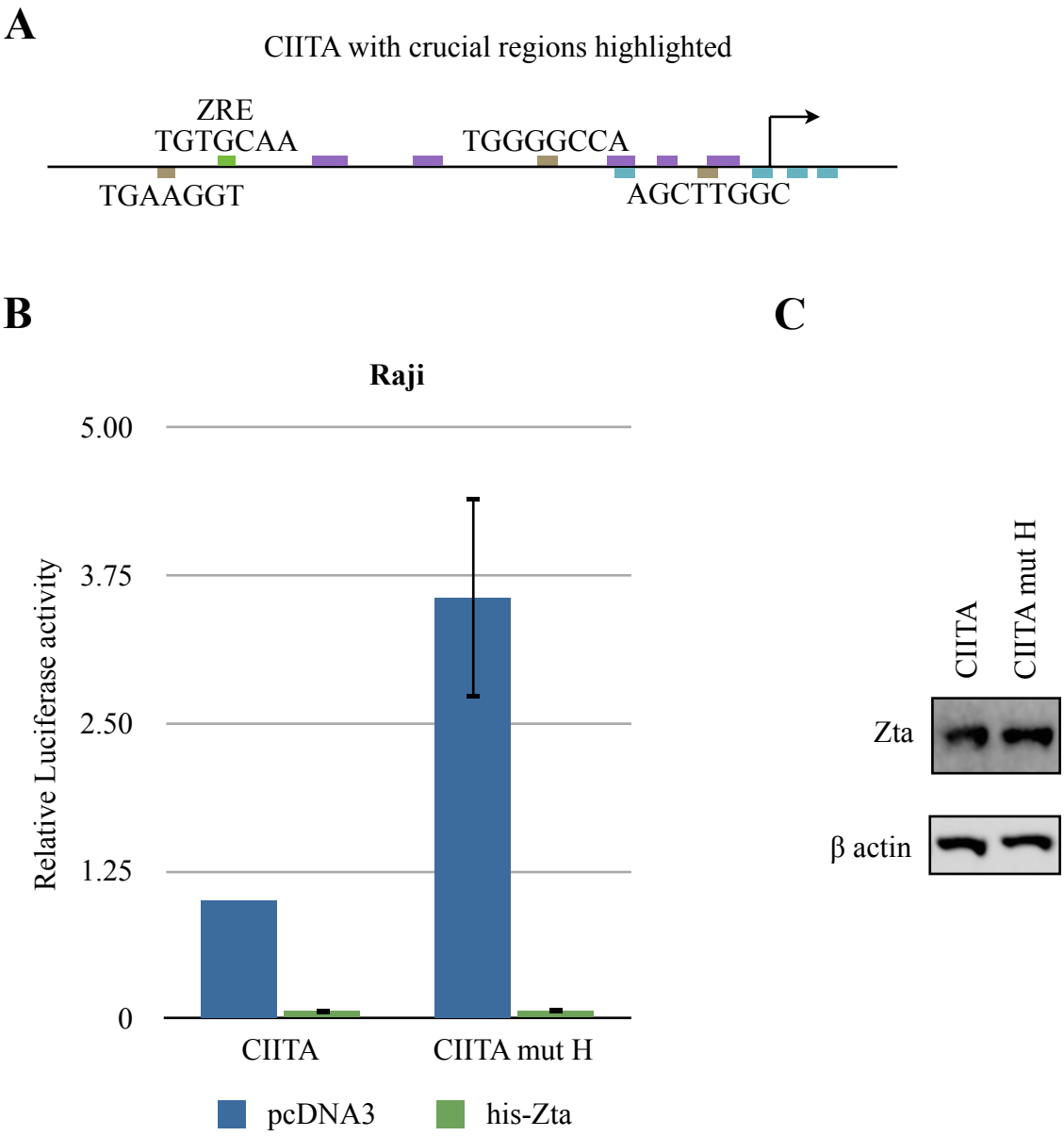


Figure 5.5 Repression of CIITA mutant H.
A. Schematic representation of the CIITA promoter with the crucial regions that were mutated in CIITA mut H. The ZRE shown in green was mutated to 'GGTTCAT', same as in Li et al. (2009). The regions identified by promoter comparison are shown in brown and were mutated according to the rule: G=>T; C=>A; A=>C; T=>G. **B.** Raji cells were transfected with his-Zta or empty vector (pcDNA3) and the CIITA or CIITA mutant H and processed according to Luciferase assay protocol. The values were adjusted to total protein concentration and normalised to pcDNA3+CIITA. Error bars indicate standard deviation; data from two biological replicates. **C.** Western blot analysis of Zta protein levels using BZ1 and anti-β actin antibodies.

construct was designated as CIITA mutant H. Unfortunately, the identified regions were not linked to the pIII down-regulation by Zta. As data show [Figure 5.5 (B)], the CIITA mut H construct was repressed to the same level as the wild type CIITA (-288+52) by his-Zta in Raji cells. Interestingly, the basal promoter activity of CIITA mut H was ~3.5x that of CIITA (-288+52) which points at a previously unreported possible repressive element in the CIITA promoter sequence that was mutated in the CIITA mut H. Alternatively, the mutation could have created new transcription factor binding sites that account for the increase in the promoter activity.

5.2.4 Searching for a Zta binding transcription factor.

To identify any potential factors involved in the regulation of the pIII that were also reported to bind Zta, the UCSC genome browser was used. The ENCODE ChIP-seq data overlying the pIII of *CIITA* was analysed for any potential targets [Figure 5.6]. Out of the proteins listed in Figure 5.6, NF-κB was singled out, as it was previously reported to bind Zta. A PROMO (http://alggen.lsi.upc.es) search to extract the potential transcription factor binding sites found in the pIII -214+52 region was then performed. The CTTAGCTTGG sequence was identified as the binding site for NF-κB. It was therefore decided to mutate this sequence [Figure 5.7], to analyse the effect on Zta mediated repression.

5.2.5 Systematic approach to CIITA promoter mutation.

In addition to the targeted mutagenic approach described above (in 5.2.4), a systematic mutation of the *CIITA* pIII region was also performed. Each part of the CIITA -214+52 was sequentially mutated as shown in Figure 5.7. The constructs were designed, ordered and made as described earlier. The data [Figure 5.8] confirmed the

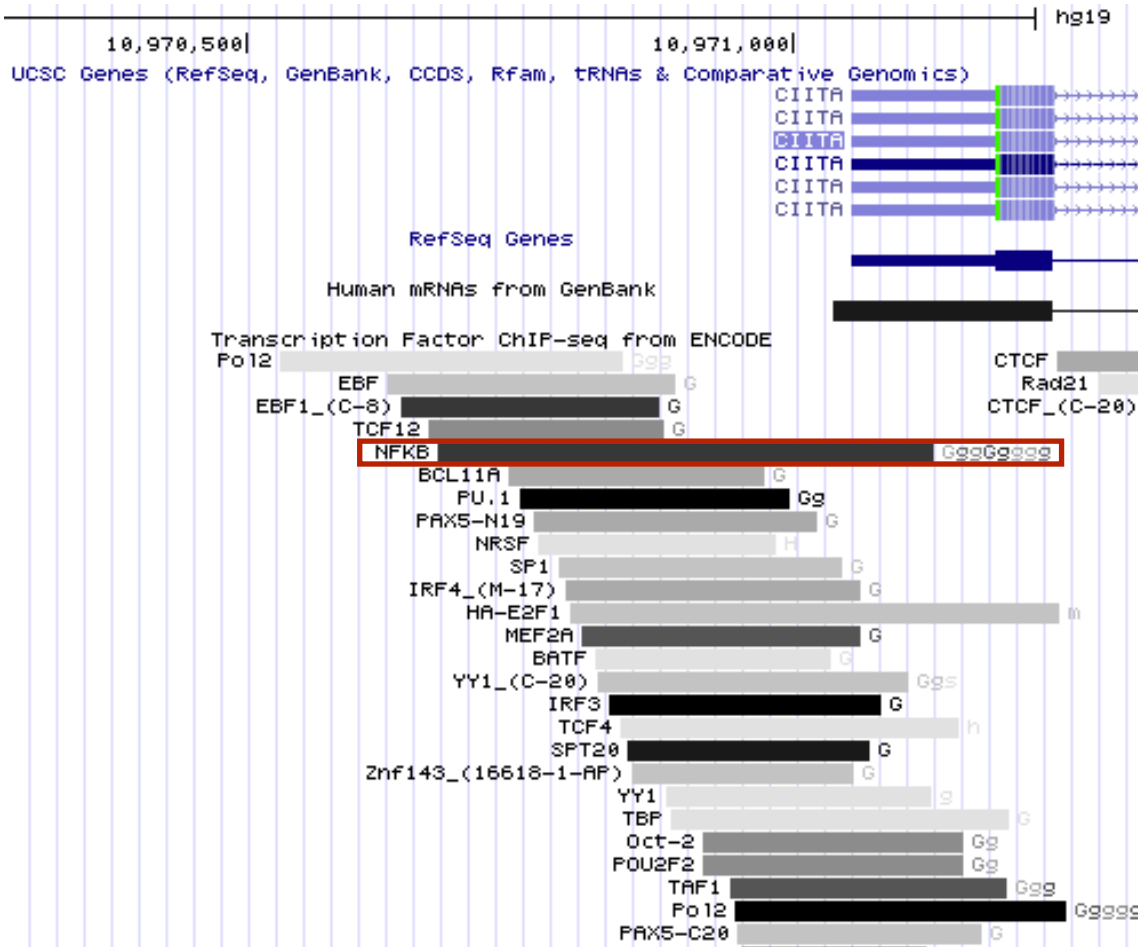


Figure 5.6 Identification of novel binding proteins.
Searching UCSC genome browser for ENCODE ChIP-seq identified binding proteins to the pIII promoter region. NF-κB is highlighted in red, as Zta was shown to previously interact with the p65 subunit of this transcription factor.

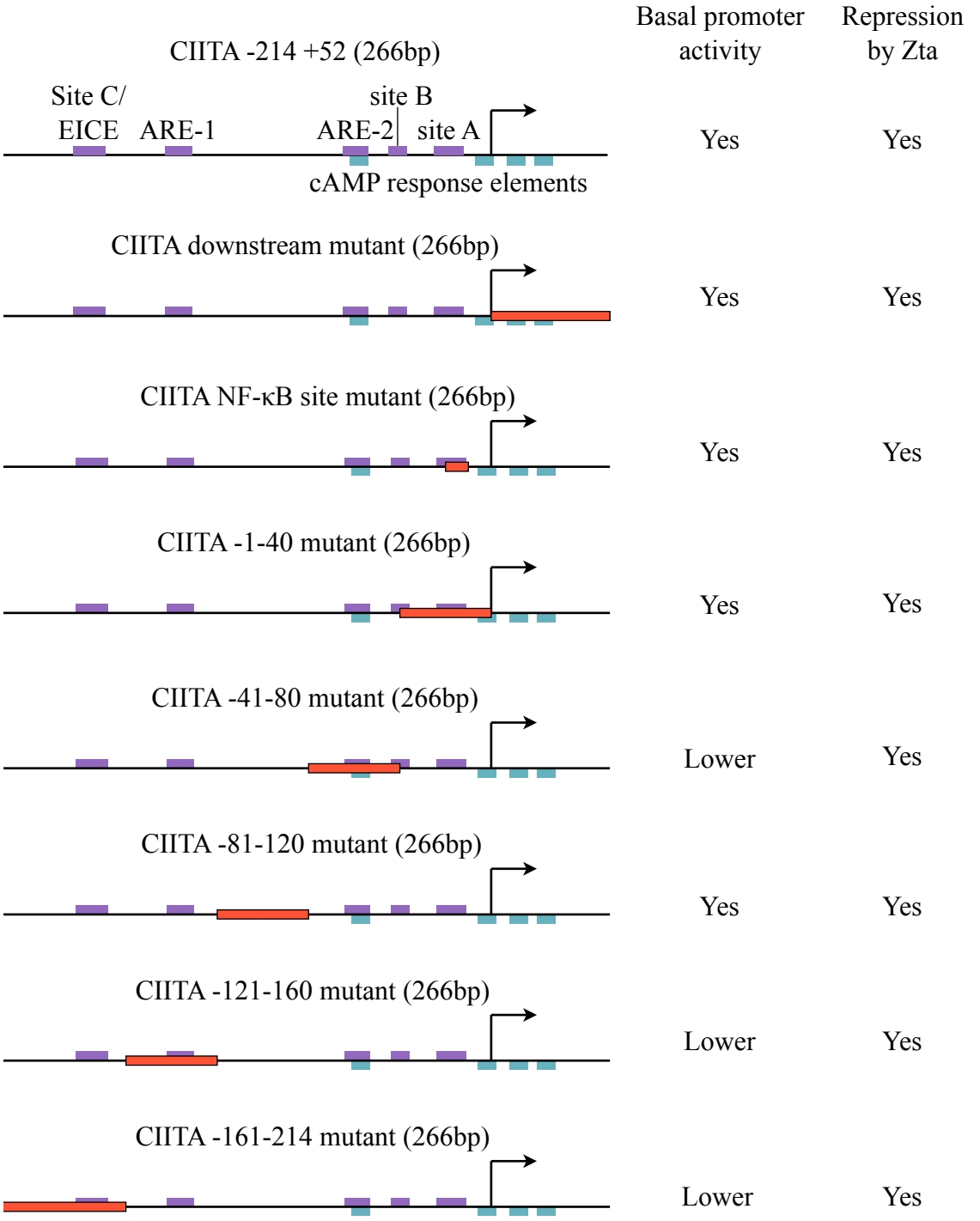


Figure 5.7 CIITA -214+52 mutants.
Schematic representation of the mutants made. The red blocks represent the mutated region according to the rule mentioned in figure 5.4 (G=>T; C=>A; A=>C; T=>G). Downstream mutant has the entire TSS downstream region mutated (52nt). The NF-κB site mutant has a predicted site (CTTAGCTTGG) 22bp upstream of TSS mutated. The rest of the mutants have the corresponding TSS upstream regions modified. The summary of results form Figure 5.8 are shown.

importance of previously published DNA sequences within pIII. CIITA -41-80 mut, modified the ARE-2 and site B, CIITA -121-160 mut had the ARE-1 region mutated and CIITA -161-214 mut lost the site C sequence. All of these constructs had a significantly lower promoter activity compared to CIITA -214+52 [Figure 5.8]. Nonetheless, his-Zta was able to repress every single mutant made [Figure 5.8], even when the basal promoter activity was diminished, including the NF-κB binding site CIITA mutant. This data suggests that Zta has a general effect on the *CIITA* promoter and is not DNA sequence specific. Additional data show that the effect observed is promoter specific. Indeed, the *BHLF1* promoter was activated in Raji cells [Figure 5.9 (A)], whilst the activity of SV40 promoter placed in a pGL3 enhancer vector (but called pGL3 control) was not significantly affected by his-Zta in the same cell system [Figure 5.9 (B)]. Together the data suggested that Zta had a general repressive effect on the pIII of *CIITA*, which was not due to a global shut off in transcription in Raji cells.

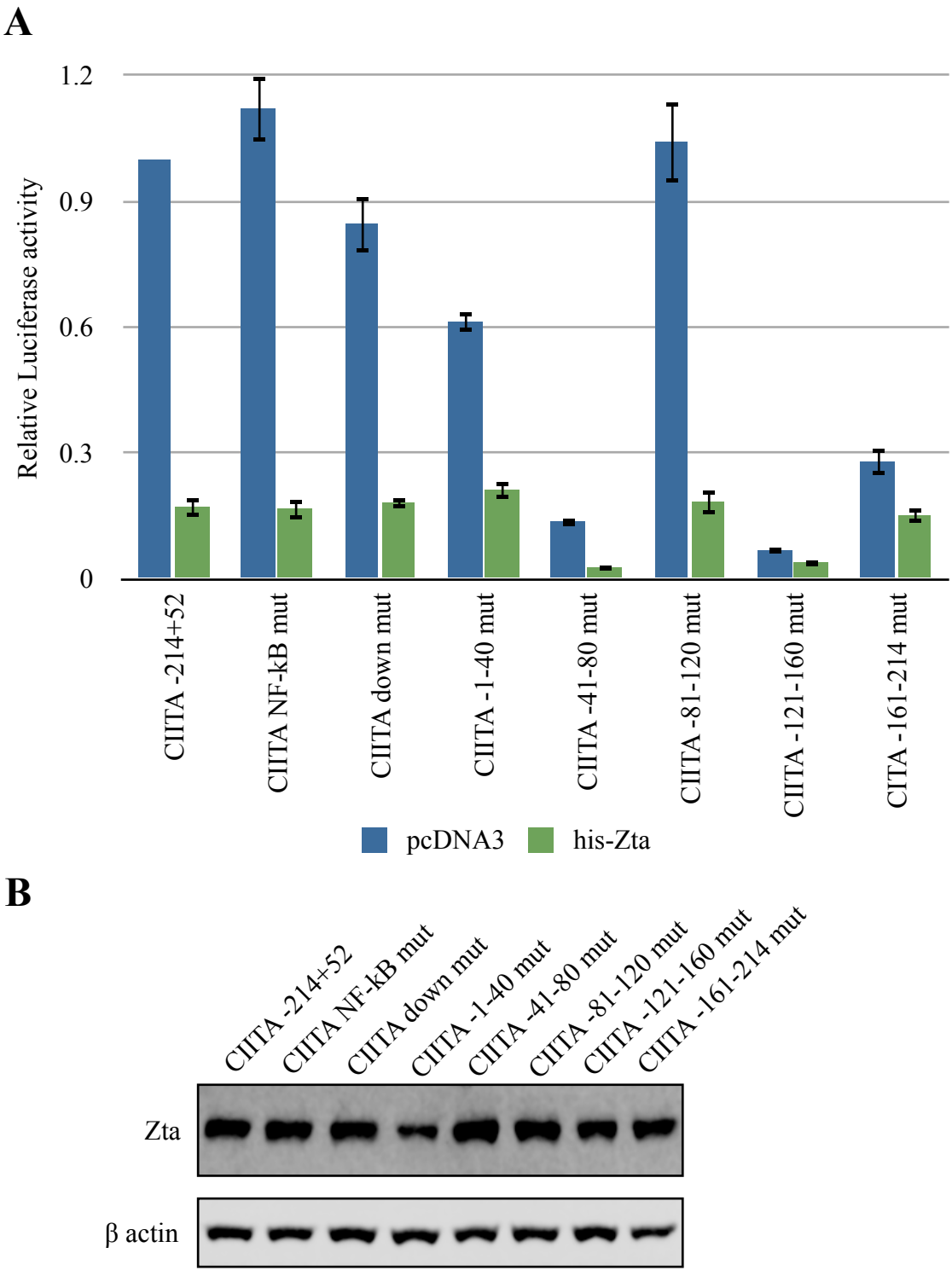


Figure 5.8 Repression by Zta is not sequence specific.
Raji cells were transfected with his-Zta or empty vector (pcDNA3) and the corresponding CIITA -214+52 mutants and processed according to Luciferase assay protocol. **A.** The Luciferase data, adjusted to total protein concentration and normalised to pcDNA3+CIITA p50 mut. Error bars indicate standard deviation; data from three technical replicates. **B.** Western blot analysis using BZ1 (Zta) and anti-β actin Ab.

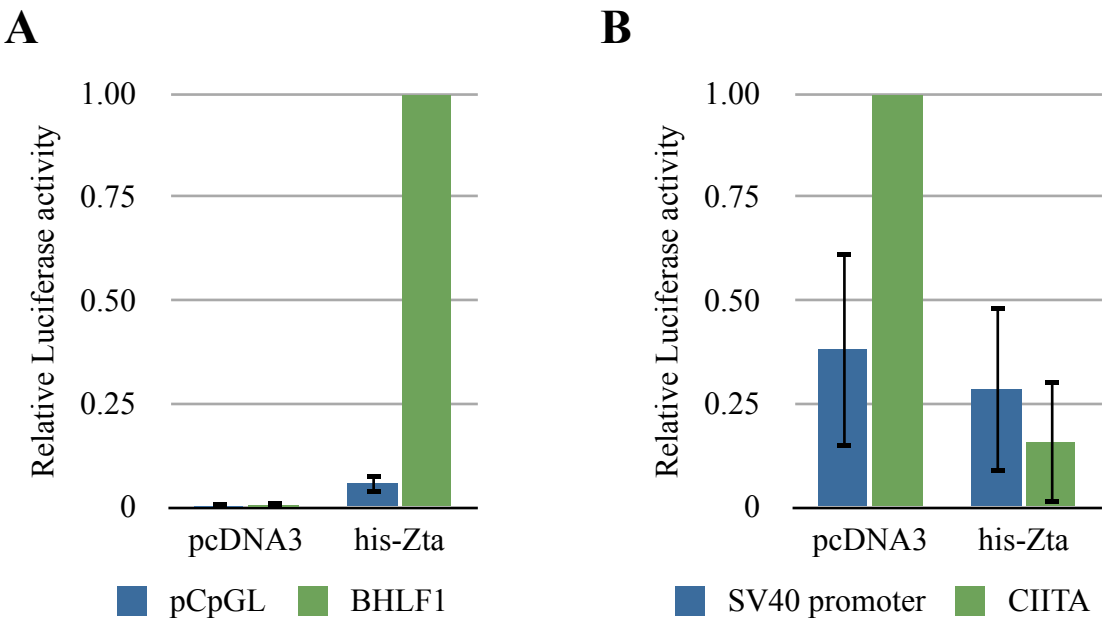


Figure 5.9 Zta does not globally repress transcription in Raji cells.
A. Raji cells were transfected with his-Zta or empty vector (pcDNA3) together with empty pCpGL or pCpGL+BHLF1 promoter. After 48h the samples were harvested according to Luciferase assay protocol. The values were adjusted to total protein concentration and normalised to his-Zta+BHLF1. Error bars indicate standard deviation; data from three biological replicates. **B.** Raji cells were transfected with his-Zta or empty vector (pcDNA3) together with SV40 promoter containing vector (pGL3 control) or the CIITA (-288+52) promoter (in a pGL3 enh. vector). After 48h the samples were harvested according to Luciferase assay protocol. The values were adjusted to total protein concentration and normalised to pcDNA3+CIITA. Error bars indicate standard deviation; data from three biological replicates.

5.2 Chapter discussion.

CIITA plays a central role in the expression of MHC class II. Because of its importance in the adaptive immune response, CIITA is tightly spatiotemporally regulated. Indeed, the complexity of CIITA control is evident at the transcriptional level (LeibundGut-Landmann et al., 2004). The *CIITA* gene has four different promoters, which govern protein expression in different cell types. Promoter III (pIII) is the primary site of *CIITA* transcription initiation in B lymphocytes, and as such is targeted by EBV, providing a mechanism by which the virus can mediate inhibition of *CIITA* (Li et al., 2009; Muhlethaler-Mottet et al., 1997). A number of regulatory DNA sequences that can bind various transcription factors have been identified and validated in pIII. Moreover, a ZRE found within the pIII promoter region was reported to be essential for Zta mediated downregulation of *CIITA* (Li et al., 2009). Nevertheless, the data in the previous chapter showed that another route of *CIITA* expression inhibition must exist, independent of the DNA binding ability of Zta. To investigate this further, a mutagenic approach of the pIII promoter was undertaken.

Modifying the *CIITA* TSS proximal ZRE to a binding nonsense sequence, or substituting it for a *BHLF1* ZRE, showed similar levels of repression as the wild type promoter in the presence of Zta. Additionally, truncation mutants of pIII, all of which lacked the ZRE sequence were also repressed by Zta. This data validates the observation from the previous chapter at a promoter level, and together the results show that the DNA binding ability of Zta is not required for all of the repression of CIITA.

Since the repression of pIII can be mediated independently of the Zta ZIP domain and does not require direct DNA binding, the Zta response must be mediated via a protein-protein interaction with an unknown transcription factor involved in the expression of *CIITA*. Several proteins have been implicated in the promoter control of pIII: TEF-2, NF-1, OTF-1, CREB, CBP, PU.1, IRF-4 and E-47 (Ghosh et al., 1999; van der Stoep et al., 2004; van der Stoep et al., 2002). Out of these transcription factors only CREB and CBP have been shown to directly bind Zta. Nonetheless, because the Zta 199ter mutant lacks the amino acids 214 and 218 needed for binding with CBP and residue 200 that is required for interaction with CREB (Adamson and Kenney, 1999), it is unlikely that these transcription factors are involved in the Zta inhibition of the *CIITA* promoter.

To identify the sites of potential Zta binding partners, several *in silico* techniques coupled with a DNA mutagenesis approach were employed: promoter comparison, database analysis and systematic pIII mutagenesis. Recent advances by Bergbauer et al. (2010) indicate that other lytic genes might be downregulated by Zta. The identified promoters include that of *BSLF1, BBLF2/3* and *BSLF2/BMLF1* genes (Bergbauer et al., 2010). Nucleotide sequences within these promoters that are shared with pIII of *CIITA* were singled out with the help of the PromoterWise tool and subsequently mutated. However, the generated construct was still repressed by Zta. Interestingly, altering these regions allowed for a higher basal activity for pIII, indicating that previously unreported negative regulatory elements within pIII might exist.

A genome browser ENCODE ChIP-seq scrutiny of the pIII region revealed that apart from the documented transcription factors mentioned above, a whole range of other proteins can bind the CIITA promoter region. Out of these proteins, NF-kB is known to directly interact with Zta (Gutsch et al., 1994). The transcription factor position weigh matrix PROMO tool allowed to pinpoint the predicted NF-kB DNA binding site within the cloned pIII construct. The mutation of this sequence did not however alter Zta's ability to downregulate CIITA, thereby ruling out the role of NF-kB in this process.

Finally, the -214+52 pIII was sequentially mutated in order to determine a potential DNA binding region of a transcription factor through which Zta might repress the *CIITA* gene. The data revealed that -161 to -214, -121 to -160 and -41 to -80 fragments were all essential for the basal promoter activity of pIII. This is most likely due to the location of EICE, ARE-1 and ARE-2 sites within these sequences. Surprisingly, Zta was able to repress all of the mutated constructs, even at lower basal activity. These findings suggest that rather than acting through a transcription factor, Zta interacts with a non-DNA binding co-regulator that acts at multiple sites within the pIII promoter.

6. General discussion.

The ability to establish a dormant latent phase during which a limited number of viral genes are expressed, together with the immune response modulating capabilities of these proteins has allowed EBV to become one of the most successful viruses known to infect humans (Ning, 2011). Additionally, EBV has developed immune evasion strategies during the lytic cycle. This is particularly important, as the lytic phase is characterised by the expression of a wide range of proteins that can elicit an immune response. Studies of donor CD4+ T cells have shown that most tested lytic antigens can cause a CD4+ T cell response, independent of the stage of the lytic phase (Long et al., 2011). CD8+ cytotoxic T cells, on the other hand, primarily recognise the two immediate early proteins, Zta and Rta, as well as some early, but not late lytic proteins. This biased response is not due to the avidity of lytic antigens for the surface receptor, but is thought to happen because as the lytic cycle progresses, the repression of MHC class I is initiated, thereby inhibiting the activation of CD8+ T cells by antigens expressed in the later stages of the lytic cycle (Pudney et al., 2005). The evident MHC class I inhibition has skewed the research focus towards investigating the EBV suppression of this pathway of antigen presentation, rather than lytic downregulation of MHC class II by the virus (involved in CD4+ response). This might also be the case because CD8+ T cell response historically was thought to play the main role in eliminating viral infections. Cytotoxic CD8+ T cells induce apoptosis in the infected cells, while helper T cells were for a long time thought to be just accessory in combating the viral infection, by promoting the growth of CD8+ T cells and antibody secreting B cells, through cytokine secretion (Heller et al., 2006; Zuo and Rowe, 2012). Nonetheless, recent studies have shown that CD4+ T cells that recognise lytic antigens can also have a direct cytolytic effect on EBV

infected LCLs (Landais et al., 2004; Long et al., 2011). Moreover, a number of EBV lytic proteins have been identified that can interfere with the MHC class II presentation pathway, including Zta inhibition of the master regulator of MHC class II, CIITA (Li et al., 2009).

The main focus of this study was to investigate the mechanism of action of Zta dependent repression of the *CIITA* pIII promoter. The results have shown that Zta can downregulate *CIITA* expression in an EBV-free cell line, AK31. This is an important finding, as it implies that other viral proteins are not required for Zta mediated repression of *CIITA*. Nevertheless, the observation that VPK can inhibit SUMOylation of Zta, together with the data from SUMO-Zta chimeras which were severely impaired for *CIITA* downregulation, suggests that VPK might play a role in promoting immune evasion through Zta. Therefore, even though EBV factors are not required, they might still regulate and fine tune Zta's ability to affect MHC class II expression.

Most surprisingly however, this study has demonstrated that apart from the DNA binding dependent repression of *CIITA*, reported by Li et al. (2009), another route of Zta mediated *CIITA* downregulation exists, independent of direct Zta-DNA interaction. The experiments have shown that neither the DNA binding incapable 199ter Zta truncation, nor the ZRE elimination from the *CIITA* pIII promoter, could abolish inhibition of this gene by Zta. Since the 199ter mutant is missing the ZIP region required for dimerisation, the action on *CIITA* must be effected by monomeric forms of Zta. On the other hand, it was revealed that the TA domain of Zta is essential for *CIITA* repression. Even though there are no known catalytic functions associated with the TA domain, this

region has been previously implicated in mediating protein-protein interactions. Taken together, the data indicate that rather than directly binding to DNA, monomeric Zta achieves *CIITA* repression via binding to other proteins.

There are a number of documented factors that were shown to regulate the pIII promoter of *CIITA*: TEF-2, NF-1, OTF-1, CREB, CBP, PU.1, IRF-4 and E-47 (Ghosh et al., 1999; van der Stoep et al., 2004; van der Stoep et al., 2002). In this study a targeted and a systematic mutagenesis approach of the pIII were employed to identify a binding site associated with a transcription factor though which Zta might inhibit *CIITA* expression. The data revealed that even though some DNA regions were involved in basal promoter activity, Zta could inhibit all pIII mutants made. These results suggest that Zta does not repress *CIITA* through interaction with a transcription factor that binds to a single site in the pIII. The only transcription factor known to bind at multiple sites in the studied pIII region is CREB (van der Stoep et al., 2002). However, since Zta 199ter lacks the amino acid 200 that is required for protein protein interaction with CREB (Adamson and Kenney, 1999), it is unlikely that Zta inhibits *CIITA* transcription though this protein. The ENCODE ChIP seq data of the pIII region, evaluated in this study, highlights that apart from the well documented and validated transcription factors, there are a number of other proteins that can bind to pIII. This shows that our understanding of the pIII regulation is far from complete. Therefore, a novel, yet unknown transcription factor could exist that binds at multiple sites within the pIII of *CIITA,* through which Zta might act. Alternatively, Zta could bind and inactivate several transcription factors involved in the activation of *CIITA* transcription.

Another group of proteins through which Zta might act to repress *CIITA*, are transcriptional coactivators. These proteins play an essential part in initiating eukaryotic transcription, providing the cells with mechanisms to control gene expression in a tissue specific and temporal manner. Coactivators are recruited to promoters by transcription factors as they do not possess DNA binding abilities themselves. Once at the DNA, coactivators can act in several different ways to help initiate gene expression. First class, are coactivators that contribute to the stabilisation of the RNA polymerase II complex bound to DNA. Second class, are ATP-dependent chromatin remodelling complexes that alter the structure of nucleosomes to allow transcription. The third class of coactivators are enzymes that modify histone tails to create euchromatin needed for efficient gene expression (Naar et al., 2001; Rosenfeld et al., 2006). Some coactivators require binding to several transcription factors to synergistically activate a promoter. The best example of this is the CIITA protein itself. CIITA is a histone acetyltransferase that contains a GTP binding domain (Raval et al., 2001). Recruitment of CIITA to the DNA requires the binding of at least three different transcription factors: RFX, NF-Y and CREB (Zhu et al., 2000). Mutating the X1, X2 or Y DNA motifs, that are the binding sites of these proteins in the MHC class II promoter, can severely impair CIITA recruitment and subsequent promoter activation (Masternak et al., 2000). Sequestration of an as yet unknown coactivator that can act through several transcription factors in the *CIITA* promoter, in a manner similar to the CIITA protein, could provide the mechanism by which Zta inhibits *CIITA* gene expression.

To identify the protein through which Zta represses the *CIITA* promoter, several biochemical techniques could be employed. The first step would be to narrow down the

exact protein domain of Zta involved in mediating the inhibition of *CIITA* transcription. This was attempted in the present study, however, expression of lower molecular weight forms of Zta in Raji cells remained elusive. It is possible that truncating Zta affects the protein's stability and turnover in the cells. To overcome this, a protein tag such as GST or GFP could be fused in front of these Zta constructs. Once the minimal region is established, a protein pull-down could be attempted to co-purify associated complexes. Followed by mass spectrometry analysis, this technique might yield a number of co-association partners. To determine which of the identified proteins play a role in the expression of the pIII promoter, a ChIP assay coupled with qPCR could be undertaken.

Understanding how EBV evades the immune response is critical for developing future therapeutic targets. The present study has shed a light on the process of Zta down-regulation of the *CIITA* expression, although the exact mechanism of action remains to be determined, and further investigations are needed.

7. Supplementary information.

7.1 Sequences of CIITA promoter constructs.

7.1.1 CIITA (-388+52).

GGTACCAGTGCGGTTCCATTGTGATCATCATTTCTGAACGTCAGACTGTTGAA
GGTTCCCCCAACAGACTTTCTGTGCAACTTTCTGTCTTCACCAAATTCAGTCC
ACAGTAAGGAAGTGAAATTAATTTCAGAGGTGTGGGGAGGGCTTAAGGGAG
TGTGGTAAAATTAGAGGGTGTTCAGAAACAGAAATCTGACCGCTTGGGGCCA
CCTTGCAGGGAGAGTTTTTTTGATGATCCCTCACTTGTTTCTTTGCATGTTGG
CTTAGCTTGGCGGGCTCCCAACTGGTGACTGGTTAGTGATGAGGCTAGTGAT
GAGGCTGTGTGCTTCTGAGCTGGGCATCCGAAAAGCTT

The sequence of the original *CIITA* pIII cloned promoter region (-388+52) is shown. RE sites, KpnI and HindIII, are highlighted in blue and the ZRE in green colour.

7.1.2 CIITA null ZRE.

GGTACCAGTGCGGTTCCATTGTGATCATCATTTCTGAACGTCAGACTGTTGAA
GGTTCCCCCAACAGACTTTCCCCCCTTCTTTCTGTCTTCACCAAATTCAGTCC
ACAGTAAGGAAGTGAAATTAATTTCAGAGGTGTGGGGAGGGCTTAAGGGAG
TGTGGTAAAATTAGAGGGTGTTCAGAAACAGAAATCTGACCGCTTGGGGCCA
CCTTGCAGGGAGAGTTTTTTTGATGATCCCTCACTTGTTTCTTTGCATGTTGG
CTTAGCTTGGCGGGCTCCCAACTGGTGACTGGTTAGTGATGAGGCTAGTGAT
GAGGCTGTGTGCTTCTGAGCTGGGCATCCGAAAAGCTT

The sequence of the ‘CIITA null ZRE’ (-388+52) is shown. RE sites, KpnI and HindIII, are highlighted in blue and the mutated ZRE in green colour.

7.1.3 CIITA ZRE 3/4.

GGTACCAGTGCGGTTCCATTGTGATCATCATTTCTGAACGTCAGACTGTTGAA
GGTTCCCCCAACAGACTTTCTGACACACTTTCTGTCTTCACCAAATTCAGTCC
ACAGTAAGGAAGTGAAATTAATTTCAGAGGTGTGGGGAGGGCTTAAGGGAG
TGTGGTAAAATTAGAGGGTGTTCAGAAACAGAAATCTGACCGCTTGGGGCCA
CCTTGCAGGGAGAGTTTTTTTGATGATCCCTCACTTGTTTCTTTGCATGTTGG
CTTAGCTTGGCGGGCTCCCAACTGGTGACTGGTTAGTGATGAGGCTAGTGAT
GAGGCTGTGTGCTTCTGAGCTGGGCATCCGAAAAGCTT

The sequence of the ‘CIITA ZRE 3/4’ (-388+52) is shown. RE sites, KpnI and HindIII, are highlighted in blue colour, and the ZRE substituted from the BHLF1 promoter ZRE 3/4 is shown in green colour.

7.1.4 CIITA -214+52.

GGTACCCTTTCTGTCTTCACCAAATTCAGTCCACAGTAAGGAAGTGAAATTA
ATTTCAGAGGTGTGGGGAGGGCTTAAGGGAGTGTGGTAAAATTAGAGGGTGT
TCAGAAACAGAAATCTGACCGCTTGGGGCCACCTTGCAGGGAGAGTTTTTTT

GATGATCCCTCACTTGTTTCTTTGCATGTTGGCTTAGCTTGGCGGGCTCCCAA
CTGGTGACTGGTTAGTGATGAGGCTAGTGATGAGGCTGTGTGCTTCTGAGCT
GGGCATCCGAAAAGCTT

The sequence of the ‘CIITA -214+52’ is shown. RE sites, KpnI and HindIII, are highlighted in blue colour.

7.1.5 CIITA -214+44.

GGTACCCTTTCTGTCTTCACCAAATTCAGTCCACAGTAAGGAAGTGAAATTA
ATTTCAGAGGTGTGGGGAGGGCTTAAGGGAGTGTGGTAAAATTAGAGGGTGT
TCAGAAACAGAAATCTGACCGCTTGGGGCCACCTTGCAGGGAGAGTTTTTTT
GATGATCCCTCACTTGTTTCTTTGCATGTTGGCTTAGCTTGGCGGGCTCCCAA
CTGGTGACTGGTTAGTGATGAGGCTAGTGATGAGGCTGTGTGCTTCTGAGCT
GGGAAGCTT

The sequence of the ‘CIITA -214+44’ is shown. RE sites, KpnI and HindIII, are highlighted in blue colour.

7.1.6 CIITA -199+52.

GGTACCAAATTCAGTCCACAGTAAGGAAGTGAAATTAATTTCAGAGGTGTGG
GGAGGGCTTAAGGGAGTGTGGTAAAATTAGAGGGTGTTCAGAAACAGAAAT
CTGACCGCTTGGGGCCACCTTGCAGGGAGAGTTTTTTTGATGATCCCTCACTT
GTTTCTTTGCATGTTGGCTTAGCTTGGCGGGCTCCCAACTGGTGACTGGTTAG
TGATGAGGCTAGTGATGAGGCTGTGTGCTTCTGAGCTGGGCATCCGAAAAGC
TT

The sequence of the ‘CIITA -199+52’ is shown. RE sites, KpnI and HindIII, are highlighted in blue colour.

7.1.7 CIITA -199+44.

GGTACCAAATTCAGTCCACAGTAAGGAAGTGAAATTAATTTCAGAGGTGTGG
GGAGGGCTTAAGGGAGTGTGGTAAAATTAGAGGGTGTTCAGAAACAGAAAT
CTGACCGCTTGGGGCCACCTTGCAGGGAGAGTTTTTTTGATGATCCCTCACTT
GTTTCTTTGCATGTTGGCTTAGCTTGGCGGGCTCCCAACTGGTGACTGGTTAG
TGATGAGGCTAGTGATGAGGCTGTGTGCTTCTGAGCTGGGAAGCTT

The sequence of the ‘CIITA -199+44’ is shown. RE sites, KpnI and HindIII, are highlighted in blue colour.

7.1.8 CIITA mutant H.

GGTACCAGTGCGGTTCCATTGTGATCATCATTTCTGAACGTCAGACTGTGTCC
TTGTCCCCCAACAGACTTTCGGTTCATCTTTCTGTCTTCACCAAATTCAGTCC
ACAGTAAGGAAGTGAAATTAATTTCAGAGGTGTGGGGAGGGCTTAAGGGAG
TGTGGTAAAATTAGAGGGTGTTCAGAAACAGAAATCTGACCGCTGTTTTAAC
CCTTGCAGGGAGAGTTTTTTTGATGATCCCTCACTTGTTTCTTTGCATGTTGG

CTTCTAGGTTAGGGCTCCCAACTGGTGACTGGTTAGTGATGAGGCTAGTGAT
GAGGCTGTGTGCTTCTGAGCTGGGCATCCGAAAAGCTT

The sequence of the 'CIITA mutant H' (-388+52) is shown. RE sites, KpnI and HindIII, are highlighted in blue colour, the ZRE substituted from the one reported in Li et al. (2009) is shown in green colour, and the mutated crucial regions after Promoter-Wise comparison are shown in red.

7.1.9 CIITA downstream mutant.

GGTACCCTTTCTGTCTTCACCAAATTCAGTCCACAGTAAGGAAGTGAAATTA
ATTTCAGAGGTGTGGGGAGGGCTTAAGGGAGTGTGGTAAAATTAGAGGGTGT
TCAGAAACAGAAATCTGACCGCTTGGGGCCACCTTGCAGGGAGAGTTTTTTT
GATGATCCCTCACTTGTTTCTTTGCATGTTGGCTTAGCTTGGCGGGCTCCCAA
CTGGTGACTGGGGCTGTCGTCTTAGCTGTCGTCTTAGTGTGTAGGAGTCTAGT
TTACGAATCCAAGCTT

The sequence of the 'CIITA downstream mutant' (214+52) is shown. RE sites, KpnI and HindIII, are highlighted in blue and the mutated region in red colour.

7.1.10 CIITA NF-κB mutant.

GGTACCCTTTCTGTCTTCACCAAATTCAGTCCACAGTAAGGAAGTGAAATTA
ATTTCAGAGGTGTGGGGAGGGCTTAAGGGAGTGTGGTAAAATTAGAGGGTGT
TCAGAAACAGAAATCTGACCGCTTGGGGCCACCTTGCAGGGAGAGTTTTTTT
GATGATCCCTCACTTGTTTCTTTGCATGTTGGCTTAGCTTGGATTTAGAAACA
CTGGTGACTGGTTAGTGATGAGGCTAGTGATGAGGCTGTGTGCTTCTGAGCT
GGGCATCCGAAAAGCTT

The sequence of the 'CIITA NF-κB' (214+52) is shown. RE sites, KpnI and HindIII, are highlighted in blue and the mutated region in red colour.

7.1.11 CIITA -1-40 mutant.

GGTACCCTTTCTGTCTTCACCAAATTCAGTCCACAGTAAGGAAGTGAAATTA
ATTTCAGAGGTGTGGGGAGGGCTTAAGGGAGTGTGGTAAAATTAGAGGGTGT
TCAGAAACAGAAATCTGACCGCTTGGGGCCACCTTGCAGGGAGAGTTTTTTT
GATGATCCCTCACTTGTTTCTTTGACGTGGTTAGGCTAGGTTATTTAGAAACC
AGTTGTCAGTTTTAGTGATGAGGCTAGTGATGAGGCTGTGTGCTTCTGAGCT
GGGCATCCGAAAAGCTT

The sequence of the 'CIITA -1-40' (214+52) is shown. RE sites, KpnI and HindIII, are highlighted in blue and the mutated region in red colour.

7.1.12 CIITA -41-80 mutant.

GGTACCCTTTCTGTCTTCACCAAATTCAGTCCACAGTAAGGAAGTGAAATTA
ATTTCAGAGGTGTGGGGAGGGCTTAAGGGAGTGTGGTAAAATTAGAGGGTGT
TCAGAAACAGAAATCTGACCGCTTGGGGCCACCTTGACTTTCTCTGGGGGGG

TCGTCGAAAGACAGGTGGGAGGGTCATGTTGGCTTAGCTTGGCGGGCTCCCA
ACTGGTGACTGGTTAGTGATGAGGCTAGTGATGAGGCTGTGTGCTTCTGAGC
TGGGCATCCGAAAAGCTT

The sequence of the ‘CIITA -41-80’ (214+52) is shown. RE sites, KpnI and HindIII, are highlighted in blue and the mutated region in red colour.

7.1.13 CIITA -81-120 mutant.

GGTACCCTTTCTGTCTTCACCAAATTCAGTCCACAGTAAGGAAGTGAAATTA
ATTTCAGAGGTGTGGGGAGGGCTTAAGGGAGTGTGGTAAAATTAGAGGTGTG
GACTCCCACTCCCGAGTCAATAGGTTTTAACAAGGTCAGGGAGAGTTTTTTT
GATGATCCCTCACTTGTTTCTTTGCATGTTGGCTTAGCTTGGCGGGCTCCCAA
CTGGTGACTGGTTAGTGATGAGGCTAGTGATGAGGCTGTGTGCTTCTGAGCT
GGGCATCCGAAAAGCTT

The sequence of the ‘CIITA -81-120’ (214+52) is shown. RE sites, KpnI and HindIII, are highlighted in blue and the mutated region in red colour.

7.1.14 CIITA -121-160 mutant.

GGTACCCTTTCTGTCTTCACCAAATTCAGTCCACAGTAAGGAAGTGAAATTA
ATTTCAGATTGTGTTTTCTTTAGGCCTTTCTGTGTTGCCCCGGCTCTTGTGTTC
AGAAACAGAAATCTGACCGCTTGGGGCCACCTTGCAGGGAGAGTTTTTTTG
ATGATCCCTCACTTGTTTCTTTGCATGTTGGCTTAGCTTGGCGGGCTCCCAAC
TGGTGACTGGTTAGTGATGAGGCTAGTGATGAGGCTGTGTGCTTCTGAGCTG
GGCATCCGAAAAGCTT

The sequence of the ‘CIITA -121-160’ (214+52) is shown. RE sites, KpnI and HindIII, are highlighted in blue and the mutated region in red colour.

7.1.11 CIITA -161-214 mutant.

GGTACCAGGGAGTGAGGACAACCCGGACTGAACACTGCCTTCCTGTCCCGG
CCGGGACTCGGTGTGGGGAGGGCTTAAGGGAGTGTGGTAAAATTAGAGGGT
GTTCAGAAACAGAAATCTGACCGCTTGGGGCCACCTTGCAGGGAGAGTTTTT
TTGATGATCCCTCACTTGTTTCTTTGCATGTTGGCTTAGCTTGGCGGGCTCCC
AACTGGTGACTGGTTAGTGATGAGGCTAGTGATGAGGCTGTGTGCTTCTGAG
CTGGGCATCCGAAAAGCTT

The sequence of the ‘CIITA -161-214’ (214+52) is shown. RE sites, KpnI and HindIII, are highlighted in blue and the mutated region in red colour.

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